MEDICAL ASPECTS OF BIOLOGICAL WARFARE



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1818
Medical Department of the Army

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The Military Surgeon, Vol XLI, No 2, 1917

Textbooks of Military Medicine

Published by the

Office of The Surgeon General
Borden Institute
US Army Medical Department Center and School
Health Readiness Center of Excellence
Fort Sam Houston, Texas

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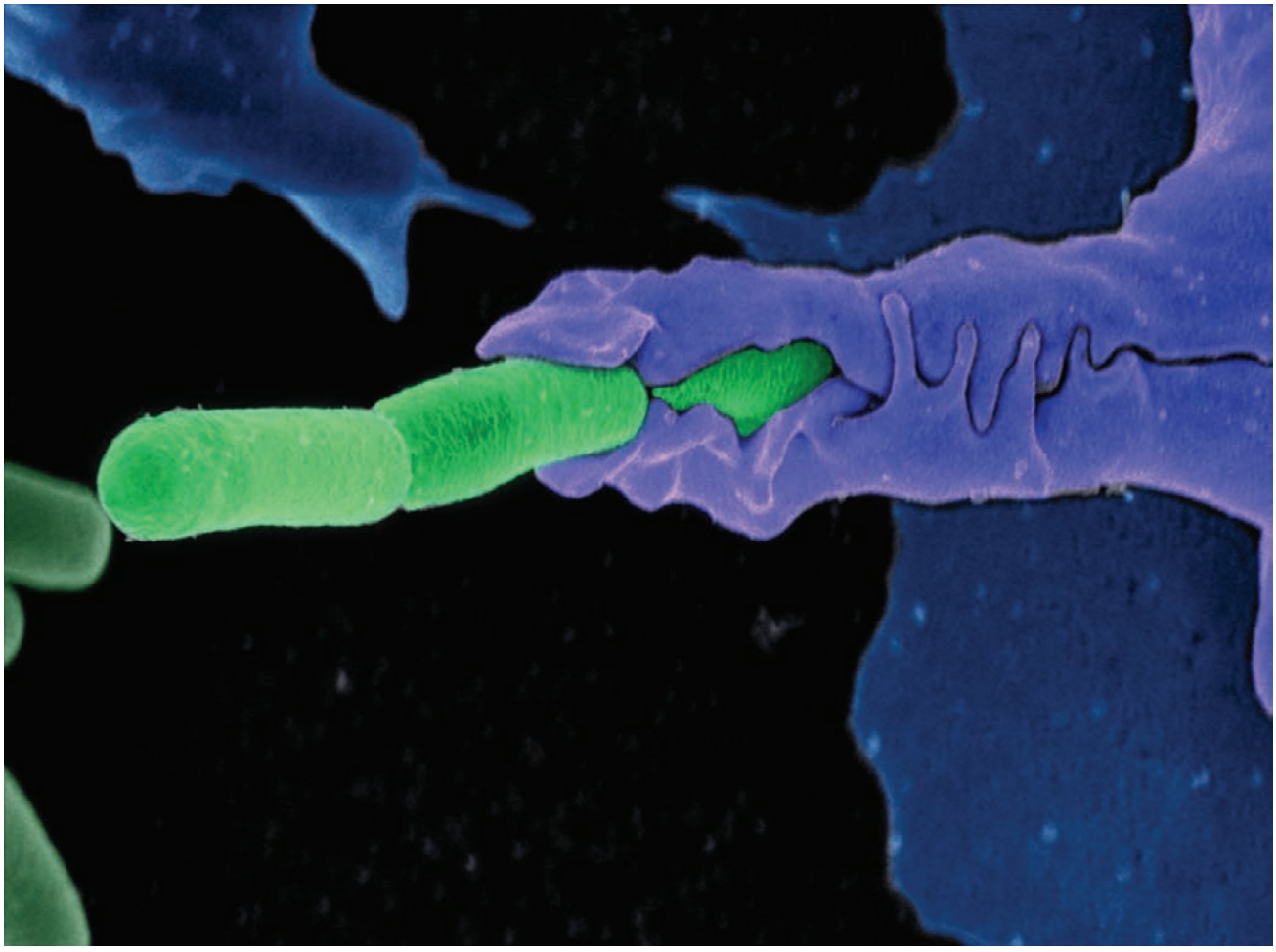
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Photograph: Courtesy of Sarah Guilman, Camenzind G. Robinson, and Arthur M. Friedlander, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

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2018

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Published by the Office of The Surgeon General
Borden Institute
US Army Medical Department Center and School
Health Readiness Center of Excellence
Fort Sam Houston, Texas

Library of Congress Cataloging-in-Publication Data

Names: Bozue, Joel, editor. | Cote, Christopher K., editor. | Glass, Pamela J., editor. | Borden Institute (U.S.), issuing body.
Title: Medical aspects of biological warfare / senior editors, Joel Bozue, Christopher K. Cote, Pamela J. Glass.
Other titles: Textbooks of military medicine.
Description: 2. | Fort Sam Houston, Texas : Office of the Surgeon General, Borden Institute, US Army Medical Department Center and School, Health Readiness Center of Excellence, 2018. | Series: Textbooks of military medicine | Includes bibliographical references and index.
Identifiers: LCCN 2017057681 | ISBN 9780160941597
Subjects: | MESH: Bioterrorism--prevention & control | Communicable Disease Control--methods | Military Medicine--methods
Classification: LCC RC971 | NLM WA 295 | DDC 616.9/8023--dc23 LC record available at Caution-https://lccn.loc.gov/2017057681

PRINTED IN THE UNITED STATES OF AMERICA

25, 24, 23, 22, 21, 20, 19, 18 5 4 3 2 1

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Foreword

The concept of national defense has been undeniably shaped by the events of September 11, 2001. The US anthrax postal attacks immediately following 9/11 forever changed our perspective of biodefense related research. More recently, the continued threat of state-sponsored events or individual extremist groups has only compounded the severity of this facet of national security. As we focus our medical efforts to succeed at the point of injury, and to optimize the success of the operating forces, the identification and preparation for biological threats has become a synergistic force multiplier.

The US Department of Defense (DoD) continues to identify potential threats and prepare for possible biological attacks by maintaining a knowledge base and by actively developing and testing novel medical countermeasures. For example, scientists at the US Army Medical Research Institute of Infectious Diseases (Fort Detrick, MD) have developed vaccines against the causative agents of anthrax, plague, ricin intoxication, botulinum intoxication, Ebola virus, and encephalitic alphaviruses. Importantly, DoD scientists partner with other federal agencies, academic institutions, and pharmaceutical companies to test and evaluate vaccines and therapeutics against many other biological threats. There is no better example of this consortium approach then the DoD's efforts during the recent Ebola outbreak in West Africa. DoD scientists and military personnel were on the ground diagnosing samples, sequencing viral genomes, and administering supportive care. Concurrently, some of the very products tested and evaluated by the DoD were deployed during the medical emergency. If it was not for DoD intervention, this outbreak had the potential to be substantially worse and spread further across Africa and around the world. Taken together, these brief examples demonstrate exactly why *Medical Aspects of Biological Warfare* needs to be maintained as an up-to-date information source.

The first edition of *Medical Aspects of Chemical and Biological Warfare* was published in 1997. A decade later, the chemical and biological aspects of this text warranted separate volumes. Thus, in 2007, *Medical Aspects of Biological Warfare* was released as a stand-alone textbook. Because of the fast-paced nature of microbiological research, new and emerging threats, and the changing policy, the authors pursued an updated version. In this third edition of the Textbooks of Military Medicine's *Medical Aspects of Biological Warfare*, the authors have gone to great lengths to address many facets of biodefense research, preparedness, and consequence management. Individual chapters are devoted to understanding the pathogenesis and disease progression associated with bacterial and viral biothreat agents, such as *Bacillus anthracis* and Ebola virus. Additionally, intoxications by toxins such as ricin are also described in detail. These chapters highlight the current state of science for these agents and toxins: they clearly underscore the importance of pursuing basic science interests in these arenas, and the importance of maintaining a core pool of subject matter experts. Without basic science efforts, our continued understanding of these threats would suffer, and knowledge gaps would grow. Accordingly, current clinical treatment protocols and regimens are also discussed throughout the textbook and offer a bridge from the basic research to the applied clinical "real-world" applications.

This textbook also examines other less apparent biodefense-related topics. *Acinetobacter baumii* is used as an example of how a drug-resistant bacterium can impact the DoD, and further demonstrates how the institutional structure and strategic planning can be used to address such threats. Additional chapters discuss Medical Management and Consequence Management, and give current perspectives on patient care and federal and local response scenarios in the event of a biological attack. This edition also describes current laboratory biosafety and biosurety philosophies that have tremendous impacts on the execution of biodefense strategies that are constantly evolving. Finally, this version of the textbook gives a nod to the history of biodefense research while also addressing new and emerging biological threats, be they natural or engineered.

The authors, subject matter expert reviewers, and editors have produced a comprehensive and thoughtful reference source for the DoD, and I am proud of the scientists, physicians, and other professionals who contributed their time and efforts to produce the final product.

Lieutenant General Nadja Y. West, MD
The Surgeon General
Commanding General, U.S. Army Medical Command

Washington, DC
March 2017

Preface

In an ever-changing and complex world, medical defense against biological pathogens must be a central pillar of our national defense strategy. Although biological warfare has been a legitimate concern for centuries, our current requirements and future operations emphasize the need for a continuing holistic approach to medical biological defense against these threats. From antiquity to the present day, agents such as *Bacillus anthracis* (etiological agent of anthrax), *Francisella tularensis* (etiological agent of tularemia), *Burkholderia mallei* (etiological agent of glanders), *Yersinia pestis* (etiological agent of plague), and *Variola* (etiological agent of smallpox) have been on the forefront of biowarfare and biodefense. With increased uncertainty associated with terrorist groups, rogue nations, and "lone wolf" individuals, the threat of biological weapons is even more relevant today.

Subject matter experts who wrote and reviewed these chapters focused on the most current data available at the time to create the most comprehensive reference source available for the US Department of Defense. Revising this textbook is important, not only to highlight the current state-of-the-art application for medical countermeasures, but also to discuss myriad current and future threats. Some of these evolving issues include the ongoing ramifications of the world's largest-ever Ebola virus disease outbreak and the impact of emerging antibiotic resistance from select bacterial pathogens. Of recent note is the emerging *B cereus* biovar *anthracis* strains isolated in Africa from fatal anthrax-like infections in chimpanzees and western lowland gorillas. These strains of *B cereus* were shown to harbor plasmids highly similar to both *B anthracis* virulence plasmids and, accordingly, were included on the US Department of Health and Human Services select agent list in 2016. These are just a few of the examples that underscore the complexities of biodefense research. Although we must remain vigilant in anticipating state-sponsored or terrorist activities, new threats are evolving in the natural world that could prove equally catastrophic to our military personnel and national interests. Preparation, cooperation, and rehearsal in accordance with the latest methodologies are the key ingredients to success in these current contexts.

I am deeply grateful for the contributions of the scientists and physicians who collaborated in this endeavor. They are nationally and internationally recognized experts in their specialties, and their dedication to updating this textbook has been invaluable. I am pleased to introduce the latest edition of *Medical Aspects of Biological Warfare*.

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October 2016

Chapter 1

HISTORICAL OVERVIEW: FROM POISONED DARTS TO PAN-HAZARD PREPAREDNESS

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INTRODUCTION

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THE US PROGRAM

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SUMMARY

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INTRODUCTION

Humans have used technology for destructive as well as beneficial purposes since prehistory. Aboriginal use of curare and amphibian-derived toxins as arrow poisons anticipated modern attempts to weaponize biological toxins such as botulinum and ricin. The derivation of the modern term "toxin" from the ancient Greek term for arrow poison, τωξικον φαρμακον (toxicon pharmicon; toxon = bow, arrow)[1,2] underscores the historical link between weaponry and biological agents.

Multiple factors confound the study of the history of biological weapons, including secrecy surrounding biological warfare programs, difficulties confirming allegations of biological attack, the lack of reliable microbiological and epidemiological data regarding alleged or attempted attacks, and the use of allegations of biological attack for propaganda and hoaxes. A review of historical sources and recent events in Iraq, Afghanistan, Great Britain, and the United States demonstrates that interest in biological weapons by state-sponsored programs, terrorists, and criminal elements is likely to continue. Humankind is witnessing a "democratization in the life sciences," in which the field is becoming industrialized and therefore making biotechnology available to an ever increasing number of people, some of whom will undoubtedly have ill intent. In addition, there are growing concerns that well-intentioned life sciences research to advance medical defenses against biological weapons agents and other highly virulent pathogens may inadvertently provide information that could be deliberately misused for biological weapons proliferation.[3]

Numerous historical examples exist of military disasters resulting from failures to adapt policy, strategy, and doctrine to offset the impact of revolutionary advances in weapons technology.[4] Biological medical defense programs, begun as narrowly focused efforts to counter a limited number of biological weapons agents, are being expanded as versatile capabilities, with a shift in emphasis from pathogen-specific approaches to capabilities-based programs to enable rapid responses to novel, potentially genetically engineered biological weapons agents. The response to biological weapons has fueled robust enterprises in basic and applied medical research, product development, manufacturing, stockpiling, infrastructure, public health policy, planning, and response capacities at local, national, and international levels.[5] Medical capabilities and biomedical research are being linked to diplomacy, commerce, education, ethics, law enforcement, and other activities to enable pan-societal sector responses to both biological weapons and the inevitable and dynamic challenges of naturally occurring emerging infectious diseases.[3] Integration of biological defense and public health programs and their mutual development must be continuous to optimize outcomes and maximize efficient utilization of limited resources, because the challenges posed by both biological weapons agents and naturally emerging pathogens are open-ended.[5]

EARLY USE

The impact of infectious diseases on military forces has been recognized since ancient times.[6,7] The use of disease as a weapon was used long before microbial pathogenesis was understood. Military leaders only knew that a cause and effect relationship existed between certain activities, locations, or exposures to victims of disease that resulted in the spread of infections that ultimately provided a military advantage. For example, an early tactic was to allow an enemy to take sanctuary in locations endemic for infectious diseases in anticipation that its troops would be afflicted, thus allowing unimpeded access of opposing armies to areas where transmission of malaria was highly likely.

Numerous anecdotal accounts exist of the attempted use of cadavers, animal carcasses, plant-derived toxins, and filth to transmit disease during antiquity through the Napoleonic era into modern times. Several examples illustrate the complex epidemiologic issues raised by biological warfare, the difficulty in differentiating epidemics resulting from biological attacks from outbreaks of disease that occur due to disruptions of war, and the adverse psychological impact of biological attacks on military operations.

During a naval battle against King Eumenes of Pergamum in 184 BCE, Hannibal ordered earthen pots filled with snakes to be hurled onto the decks of enemy ships. The pots shattered on impact, releasing live serpents among the enemy sailors. The Carthaginian victory is attributed to the ensuing panic rather than envenomation[8]; this illustrates that the psychological contagion of biological weapons may amplify their impact beyond their potential to cause organic disease.

One of the most notorious early biological warfare attacks was the hurling of cadavers over the walls of the besieged city of Caffa, a Genoese colony in the Crimea, in 1346.[9,10] After war broke out between the Genoese and the Mongols in 1343 for control of the lucrative caravan trade route between the Black Sea and the Orient, the Mongols laid siege to Caffa. The plague, later known as the Black Death, was

spreading from the Far East and reached the Crimea in 1346. The Mongols were severely afflicted and forced to abandon their siege. As a parting shot, they hurled "mountains of dead" over the city wall, probably with the use of a trebuchet, in the hope that "the intolerable stench would kill everyone inside." An outbreak of plague in the city followed. A review by Wheelis[10] suggests that the introduction of plague into the city by the cadavers—as a result of a tactically successful biological attack—may be the most biologically plausible of several competing hypotheses on the source of the outbreak. Although the predominant mode of plague transmission has been attributed to bites from infected fleas (which leave cadavers and carcasses to parasitize living hosts), modern experience (United States 1970–1995)[11] has implicated transmission from contact with infected animal carcasses in 20% of instances in which the source of the infection could be attributed. Contact with tissue and blood would have been inevitable during the disposal of hundreds or possibly thousands of cadavers. Alternatively, plague could have been introduced by imported human cases or infected rodents brought into the city through maritime trade, which was maintained during the siege. The importation of plague by a rodent-flea transmission cycle across the city wall is considered less likely because rats are sedentary and rarely venture far from their nests; it is unlikely that they would have traversed an open distance of several hundred meters between the Mongol encampment and the city walls.[10] Transmission from sylvatic to urban rodents is infrequent, at least under current ecological conditions.[12] Regardless of the portal of entry, the epidemic may have been amplified under siege conditions due to deteriorating sanitation and hygiene resulting in expansions of rats and fleas.

Smallpox was particularly devastating to Native Americans. The unintentional yet catastrophic introduction of smallpox to the Aztec empire during 1520, and its subsequent spread to Peru in advance of Pizarro's invasion of the Inca empire, played a major role in the conquest of both empires.[13] During the French and Indian Wars (1754–1763), British forces provided Native Americans with handkerchiefs and blankets contaminated with scabs from smallpox patients to transmit disease.[14–18] An epidemic of smallpox followed among the Native Americans of the Ohio River Valley. It is difficult to evaluate the tactical success of these biological attacks in retrospect because smallpox may have been transmitted during other contacts with colonists, as had previously occurred in New England and the South. Smallpox scabs are thought to have low infectivity due to the binding of virions in a fibrin matrix, and transmission by fomites has been considered less efficient than respiratory droplet transmission.[13]

THE WORLD WARS

The birth of scientific bacteriology during the 19th century provided the scientific and technical basis for modern biological weapons programs. The Hague Conventions of 1899 and 1904 outlawed the use of "poison or poisoned arms," although bacteriological weapons were not specifically addressed.[19–20] During World War I, German espionage agents reportedly infected draft animals intended for military use with *Burkholderia [Pseudomonas] mallei* and *Bacillus anthracis*.[21–23] Covert operations were reportedly conducted in Argentina, Norway, Mesopotamia, Romania, Russia, and the United States. Unsuccessful attempts were also made to cripple grain production in Spain using wheat fungus.[21]

The German biowarfare program of World War I is of special interest because it was the first program with a scientific basis; it conducted a large-scale (strategic) biological campaign, which targeted neutral nations as well as belligerents, and it targeted crops and animals instead of humans. Although German operatives thought the program was successful, confirmatory data are not available.[21]

In response to chemical warfare during World War I, the 1925 Geneva Protocol, an international protocol (for the Prohibition of the Use in War of Asphyxiating, Poisonous or Other Gases, and of Bacteriological Methods of Warfare), was formulated by the League of Nations' Conference for the Supervision of the International Trade in Arms and Ammunition. It had no verification mechanism and relied on voluntary compliance. Many of the original signatory states reserved the right to retaliatory use, making it effectively a no first-use protocol. Signatories that began research programs to develop biological weapons between World War I and II included Belgium, Canada, France, Great Britain, Italy, The Netherlands, Poland, and the Soviet Union.[24]

After defeating Russia in the 1905 Russo-Japanese War, Japan became the dominant foreign power in Manchuria, and seized full military control between September 1931 and the end of 1932. Major Shiro Ishii, a Japanese army physician, established a biological weapons laboratory in Harbin, but soon realized that his controversial involuntary human research could not be conducted freely there. Ishii moved to a secret facility at Beiyinhe, 100 km south of Harbin, and began large-scale experimentation. All research study subjects died of either experimental infection or live vivisection. These studies continued until a prisoner

riot and escape, which resulted in the closing of the facility in 1937. However, larger and more extensive facilities were subsequently built.[24]

In 1936 Ishii built Unit 731, a massive research facility 24 km south of Harbin, where a census of 200 prisoners was kept as expendable subjects of experimentation. Ultimately, more than 3,000 Chinese prisoners were killed during these experiments. Most of the evidence was destroyed at the end of the war, and in all likelihood the actual number was much greater.[24] Additional facilities included Unit 100 at Changchun, and Unit Ei 1644 in Nanking. Unit 100 was primarily a veterinary and agricultural biowarfare research unit for developing biological weapons for sabotage. Although animals and crops were the focus of most of the research, numerous human studies were also conducted, similar to those conducted by Unit 731. In addition to conducting human experimentation, Unit Ei 1644 supported Unit 731's research efforts with bacterial agent production and flea cultivation.[24]

Eleven Chinese cities were allegedly attacked during "field trials" using agents including *Yersinia pestis*, *Vibrio cholerae*, and *Shigella spp*. These attacks may have backfired because up to 10,000 Japanese soldiers reportedly contracted cholera after a biological attack on Changde in 1941.[25] The field trials were terminated in 1943, yet basic research and human experimentation continued until the end of the war.[24–26] Despite the enormously expensive program (both in terms of national treasure and human lives) and the weaponization of many agents, Japan never developed a credible biowarfare capability, mainly because of the failure to develop an effective delivery system.[17]

In contrast to Japanese efforts during World War II, a German offensive biological weapons program never materialized. Hitler reportedly issued orders prohibiting biological weapons development. Unethical experimental infections of prisoners were done primarily to study pathogenesis and develop vaccines and sulfonamides, rather than to develop biological weapons. With the support of high-ranking Nazi party officials, however, scientists began biological weapons research, but their results lagged far behind those of other countries.[27]

Polish physicians used a vaccine and a serologic test in a brilliant example of "biological defense." Knowing that inoculation with killed Proteus OX-19 would cause false-positive Weil-Felix typhus test results, physicians inoculated local populations with formalin-killed Proteus OX-19 to create serologic pseudoepidemics of typhus. Using serologic surveillance, the German army avoided areas with epidemic typhus; consequently, residents of these areas were spared deportation to concentration camps.[28] Unconfirmed allegations indicate that Polish resistance fighters used letters contaminated with *B anthracis* to cause cutaneous anthrax among Gestapo officials[21,29] and used typhus against German soldiers.[21] Czechoslovakian agents reportedly used a grenade contaminated with botulinum toxin, supplied by British Special Operations, to assassinate Reinhard Heydrich, the Nazi governor of occupied Czechoslovakia[30,31]; however, the veracity of this claim has been challenged.[23]

The perceived threat of biological warfare before World War II prompted Great Britain to stockpile vaccines and antisera, establish an emergency public health laboratory system, and develop biological weapons. "Cattle cakes" consisting of cattle feed contaminated with *B anthracis* spores were designed to be dropped from aircraft into Axis-occupied Europe to cause epizootic anthrax among livestock,[32,33] which would in turn induce famine. The cattle cakes were intended as a strategic economic weapon rather than as a direct cause of human anthrax. In addition, explosive munitions designed to aerosolize and disperse *B anthracis* spores as antipersonnel weapons were tested on Gruinard Island near the coast of Scotland in 1942. These experiments successfully caused anthrax in targeted sheep.[34] The antipersonnel weapons were not mass produced, and neither the cattle cakes nor the explosive munitions were used.[21] Great Britain continued its offensive biological warfare program during the early Cold War era in conjunction with the United States and Canada, and it performed secret open-air tests using pathogens in off-shore ocean sites near the Bahamas and Scotland.[21] Great Britain's offensive program was terminated between 1955 and 1956[35] because of budgetary constraints and reliance on nuclear deterrence.[32,33] Gruinard Island, which had been quarantined because of focal soil contamination by *B anthracis* spores following munitions testing, was decontaminated in 1986 using 2,000 tons of seawater and 280 tons of formaldehyde.[36] The United Kingdom conducts research to develop medical countermeasures at the Defence Science and Technologies Laboratories at Porton Down.

THE US PROGRAM

The US military recognized biological warfare as a potential threat after World War I. Major Leon Fox of the Army Medical Corps wrote an extensive report concluding that improvements in health and sanitation made biological weapons ineffective. In 1941, before the US entry into World War II, opinions differed

about the threat of biological warfare. Consequently, the Secretary of War asked the National Academy of Sciences to appoint a committee to study the issue. The committee concluded in February 1942 that biowarfare was feasible and the United States should reduce its vulnerability.

President Franklin D Roosevelt established the War Reserve Service (with George W Merck as director) to develop defensive measures against biological weapons. By November 1942 the War Reserve Service asked the Army's Chemical Warfare Service to assume responsibility for a secret large-scale research and development program, including the construction and operation of laboratories and pilot plants. The Army selected a small National Guard airfield at Camp Detrick in Frederick, Maryland, for the new facilities in April 1943. By summer of 1944 the Army had testing facilities in Horn Island, Mississippi (later moved to Dugway, Utah), and a production facility in Terre Haute, Indiana. No agents were produced at the Terre Haute plant because of safety concerns; simulant tests disclosed contamination after trial runs. In the only reported US offensive use of a biological weapon, the Office of Strategic Services (predecessor of the Central Intelligence Agency) used staphylococcal enterotoxin in a food-borne attack to cause an acute but self-limited illness in a Nazi party official.[37,38] Cattle cakes using *B anthracis* spores were produced at Camp Detrick and shipped to Great Britain, but were never used. The War Reserve Service was disbanded after the war and the Terre Haute plant was leased for commercial pharmaceutical production.[31] In January 1946 Merck reported to the Secretary of War that the United States needed a credible capability to retaliate if attacked with biological weapons. Basic research and development continued at Camp Detrick.

The United States learned of the extent of Japanese biological weapons research after World War II. In an action that has become controversial, Ishii and his fellow scientists were given amnesty for providing information derived from years of biological warfare research.[24]

When war broke out on the Korean peninsula in June 1950, concerns about Soviet biological weapons development and the possibility that the North Koreans, Chinese, or Soviets might resort to biological warfare resulted in an expansion of the US program. A large-scale production facility in Pine Bluff, Arkansas, was established. The plant featured advanced laboratory safety and engineering measures enabling large-scale fermentation, concentration, storage, and weaponization of microorganisms. In 1951 the first biological weapons, anticrop bombs, were produced. The first antipersonnel munitions were produced in 1954 using *Brucella suis*. The United States weaponized seven antipersonnel agents and stockpiled three anticrop agents (Table 1-1) over 26 years.[39]

Field tests using surrogate agents were conducted in the United States between 1949 and 1968, in which the general public and test subjects were uninformed. At least 239 open-air tests were conducted at several locations including the Dugway Proving Ground, Utah; remote Pacific Ocean sites; and populated areas including Minneapolis, St. Louis, New York City, San Francisco, and Eglin Air Force Base, Florida. These studies tainted the history of the offensive biological warfare program. The Special Operations Division at Camp Detrick conducted most of the field tests to study possible methods of covert attack and to examine aerosolization methods, the behavior of aerosols over large geographic areas, and the infectivity and rates of decay of aerosolized microbes subjected to solar irradiation and climatic conditions. Most tests used simulants thought to be nonpathogenic, including *Bacillus globigii*, *Serratia marcescens*, and particulates of zinc cadmium sulfide.[39,40]

In conjunction with the US Department of Agriculture (USDA), several open-air tests were conducted using anticrop agents at sites selected for safety.

TABLE 1-1

BIOLOGICAL AGENTS PRODUCED BY THE US MILITARY (DESTROYED 1971–1973)*

Lethal Agents	Incapacitating Agents	Anticrop Agents
Bacillus anthracis	*Brucella suis*	Rice blast
Francisella tularensis	*Coxiella burnetii*	Rye stem rust
Botulinum toxin	Venezuelan equine encephalitis virus	Wheat stem rust
	Staphylococcal enterotoxin B	

*Lethal and incapacitating agents were produced and weaponized. Anticrop agents were produced but not weaponized.

Open-air releases of human pathogens (*Coxiella burnetii*, *Francisella [Pasturella] tularensis*) were performed at the Dugway Proving Ground, Eglin Air Force Base, and remote Pacific Ocean sites to study viability and infectivity using animal challenge models.[21,39,40] Controversial studies included environmental tests to determine whether African Americans were more susceptible to *Aspergillus fumigatus*, as had been observed with *Coccidioides immitis*. These studies included the 1951 exposure of uninformed workers at Norfolk Supply Center in Norfolk, Virginia, to crates contaminated with *Aspergillus* spores. In 1966 the US Army conducted covert experiments in the New York City subways. Light bulbs filled with *Bacillus subtilis var niger* were dropped from subway platforms onto the tracks to study the distribution of the simulant through the subway system.[39–41] Similar tests were conducted using the ventilation system of the New York City subways and the Pentagon.

The first large-scale aerosol vulnerability test conducted in San Francisco Bay in September 1950 using *B globigii* and *S marcescens* demonstrated the public health issues of such testing.[41] An outbreak of 11 cases of nosocomial *S marcescens* (*Chromobacterium prodigiosum*) urinary tract infection occurred at the nearby Stanford University Hospital; one case was complicated by fatal endocarditis. Risk factors included urinary tract instrumentation and antibiotic exposures.[42] No similar outbreaks were reported by other San Francisco area hospitals. A panel of civilian and academic public health experts secretly convened by the Army in 1952 failed to reach a conclusion regarding the possible link between the Stanford outbreak and the testing program, but recommended that other microbes be used as simulants.[41] Public disclosure of the testing program in the *Washington Post* on December 22, 1976, and in US Senate hearings in 1977[43] resulted in harsh criticism of the continued use of *S marcescens* as a simulant after the Stanford epidemic. However, a 1977 report from the Centers for Disease Control and Prevention (CDC) concluded that in 100 outbreaks of *S marcescens* infection, none was caused by the 8UK strain (biotype A6, serotype O8:H3, phage type 678) used by the Army testing program.[44] Other reports from the 1970s postulated a link between *S marcescens* infection and the testing program; however, all clinical isolates available for strain typing were antigenically distinct from the Army test strain. In all likelihood, the 1950 Stanford *S marcescens* epidemic represents an early example of a nosocomial outbreak caused by opportunistic pathogens of low virulence complicating the use of medical devices and surgical procedures in the setting of antibiotic selection pressure.[44]

The US program developed modern biosafety technologies and procedures including protective equipment, engineering and safety measures, and medical countermeasures, including new vaccines. There were 456 occupational infections and three fatalities (two cases of anthrax in 1951 and 1958 and a case of viral encephalitis in 1964) reported at Fort Detrick during the offensive program (1943–1969).[39] The infection rate of fewer than 10 infections per million hours of work was within the contemporary National Safety Council standards; the morbidity and mortality rates were lower than those reported by other laboratories. There were 48 infections and no fatalities at the production and testing sites.[39]

In 1954 the newly formed Medical Research Unit at Fort Detrick began studies to develop vaccines and therapy to protect against biological agents. Researchers began using human volunteers in 1956 as part of a congressionally approved program called "Operation Whitecoat." This use of volunteers set the standard for ethics and human use in research. Active duty soldiers with conscientious objector status served as research subjects, and participation was voluntary with informed consent. The program concluded with the end of conscription in 1973.

Numerous unsubstantiated allegations were made during the Cold War era. During the Korean War (1950–1953), North Korean, Chinese, and Soviet officials made numerous accusations of US biowarfare attacks. Many allegations appear to be based on Chinese experiences during World War II field testing conducted by the Japanese Unit 731. Polish medical personnel were sent to China to support the communist war effort, accompanied by eastern European correspondents, who made numerous accusations based on anecdotal accounts of patients. These allegations, however, were not supported by scientific evidence. Some stories, such as the use of insect vectors to spread cholera, had dubious scientific plausibility. The North Korean and Chinese governments ignored or dismissed offers from the International Committee of the Red Cross and the World Health Organization (WHO) to conduct impartial investigations. The Soviet Union thwarted a proposal from the United States and 15 other nations to the United Nations (UN) requesting the establishment of a neutral commission for investigation. The United States admitted to having biological weapons but denied using them. The credibility of the United States may have been undermined by the knowledge of its biological weapons program and its failure to ratify the 1925 Geneva Protocol until 1975. Although unsubstantiated, these accusations resulted in a loss of international goodwill toward the United States and demonstrated the propaganda

value of biological warfare allegations, regardless of veracity.[43] Reviews of documents from former Soviet archives provide evidence that the allegations were fictitious propaganda.[45–47]

The Soviet Union accused the United States of testing biological weapons on Canadian Eskimos, resulting in a plague epidemic,[48] and of collaborating with Colombia in a biological attack on Colombian and Bolivian peasants.[49] The United States was also accused of planning to initiate an epidemic of cholera in southeastern China[50] and of the covert release of dengue in Cuba.[51] Similarly, the US allegations that Soviet armed forces and their proxies had used "yellow rain," aerosolized trichothecene mycotoxins (inhibitors of DNA and protein synthesis derived from fungi of the genus *Fusarium*) in Laos (1975–1981), Kampuchea (1979–1981), and Afghanistan (1979–1981), are widely regarded as unsubstantiated. The remote locations of the alleged attacks made intelligence investigations difficult. Western intelligence operatives never witnessed these alleged attacks, and no samples of the aerosols were recovered. Confounding factors included:

- contradictory testimonies from survivors of alleged attacks;
- discrepancies in reported symptoms;
- low disease rates in the allegedly attacked populations;
- the recovery of mycotoxin in fewer than 10% of the clinical and environmental samples submitted;
- the presence of *Fusarium* organisms as environmental commensals;
- the possible decay of toxin under prevailing environmental conditions;
- conflicting results of toxin assays from different laboratories;
- the similarity of alleged yellow rain deposits recovered from environmental surfaces to bee feces in ultrastructural appearance and pollen and mold content; and
- the natural occurrence of showers of bee feces from swarms of honey bees in the rain forests of southeast Asia.[52]

The US offensive program resulted in an understanding of the strategic nature of biological weapons. By the late 1950s assessments of the potential utility of biological weapons were mixed. In a letter from one of Dwight D Eisenhower's President's Science Advisory Council members, George Kistiakowsky, to James Killian, the chair of the council, the author made it clear that developing highly concentrated formulations of biological agents, proper handling of pathogens, and appropriate weaponization would result in cases that did not act as "normal" disease.[53] At high concentrations and in a dried formulation, biological agents had the potential for causing high mortality and morbidity. Still, questions remained about the potential to successfully use biological weapons in a controlled and reliable manner. The follow-on testing authorized by President John F Kennedy under the umbrella program of Project 112 was designed to fill in these knowledge gaps.[40,54] In the Bay of Pigs operation of 1961, military planners had developed enough interest in biological weapons that their use was contemplated. The codenamed "Marshall Plan" called for releasing incapacitating agents to attack defenders on the beach. Ultimately, the plan was scrapped and biological weapons were not used.[55]

By the late 1960s domestic and international pressures were calling for the elimination of the US offensive biological warfare program. At Dugway Proving Ground, an incident involving chemical weapons testing caused the death of 3,000 sheep. Debates about chemical and biological weapons, both for and against the development of offensive capabilities, ensued between Congress, the administration, industry, and even private citizens. In Europe draft texts of what would later become the Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological and Toxin Weapons and on their Destruction (1972 Biological Weapons Convention [BWC]) were being developed by Great Britain, Sweden, and the Soviet Union.

In May 1969 US President Richard Nixon called for an interagency review of chemical-biological warfare policies. The review was authorized as part of National Security Study Memorandum 59. The findings resulted in recommendations to President Nixon to eliminate the US offensive program and retain a defensive program.

To this end, on November 25, 1969, when visiting Fort Detrick, President Nixon announced a new US policy on biological warfare, unilaterally renouncing the development, production, stockpiling, and use of biological weapons. In explaining his decision, President Nixon stated, "Biological weapons have massive, unpredictable, and potentially uncontrollable consequences. They may produce global epidemics and impair the health of future generations."[56] Almost immediately after the statement, confusion and a potential loophole caused by the ambiguity concerning biologically derived toxins that were technically excluded from the renunciation were corrected through National Security Study Memorandum–85, "Review of Toxins Policy," which was issued on December 31, 1969.

The US Army Medical Unit was closed, and Fort Detrick and other installations in the offensive weapons program were redirected to solely develop defensive measures such as vaccines, drugs, and diagnostics. The US Army Medical Research Institute of Infectious Diseases (USAMRIID) was created with biosafety level 3 and 4 laboratories dedicated to developing medical defensive countermeasures. By May 1972 all antipersonnel agents had been destroyed, and the production facility at Pine Bluff, Arkansas, was converted into a research facility. By February 1973 all agriculture-targeted biological agents had been destroyed. Although staphylococcal entertoxin was used during World War II by Office of Strategic Services' espionage agents,[37,38] biological weapons have never been used by the US Armed Forces.[39] The Central Intelligence Agency developed weapons containing cobra venom and saxitoxin for covert operations; all records regarding their development and deployment were destroyed in 1972; all remaining toxin samples were destroyed per presidential orders after a US Senate investigation.[37] The United States signed and ratified both the 1925 Geneva Convention and the 1972 BWC, which outlaws all offensive biological weapons research, production, and possession, in 1975 (see Disarmament: The Biological Weapons Convention).

Although many welcomed the termination of the US offensive program for moral reasons, the decision was partly motivated by pragmatic considerations. Biological weapons were unnecessary for national security because of a formidable arsenal of conventional, chemical, and nuclear weapons. Although open-air simulant studies suggested that biological weapons would be effective, the potential effects of aerosols of virulent agents on targeted populations were still conjectural and could not be empirically validated for ethical and public health reasons. Despite evidence to the contrary from information obtained through the US offensive program, some still considered biological weapons to be untried, unpredictable, and potentially hazardous for the users. Field commanders and troops were unfamiliar with their use. Most importantly, the United States and allied countries had a strategic interest in outlawing biological weapons programs to prevent the proliferation of relatively low-cost weapons of mass destruction. Outlawing biological weapons made the arms race for weapons of mass destruction prohibitively expensive, given the cost of nuclear programs.[21,57]

THE SOVIET PROGRAM

Although a signatory to the 1925 Geneva Convention, the Soviet Union began a weapons development program in 1928[58] under the control of the state security apparatus, GPU (the Unified State Political Administration of the Committee of People's Commissars of the USSR). Work was initially done with typhus, reportedly with experimentation on political prisoners at Slovetsky Island in the Baltic Sea and nearby concentration camps. The program subsequently expanded to include work with the agents of Q fever, glanders, and melioidosis, and possibly tularemia and plague. Outbreaks of Q fever and tularemia among German troops are two suggested, but unconfirmed, Soviet uses of biological warfare during World War II.[59] However, the origin of epidemic tularemia during the battle of Stalingrad as a consequence of biological warfare has been challenged and attributed to natural causes and a breakdown of public health.[60] Similar outbreaks of Q fever in Axis troops in Italy, Greece, Bulgaria, and the Ukraine[61]; in Allied troops in the Mediterranean Theater[62–64]; and more recently, among Czech peacekeepers in Bosnia-Herzegovina[65] and tularemia among civilians during the Kosovo conflict[66] have been attributed to amplification of natural transmission cycles during wartime.

Stalin was forced to move his biological warfare operations out of the path of advancing German forces. Laboratories were moved to Kirov in eastern European Russia, and testing facilities were eventually established on Vozrozhdeniya Island on the Aral Sea between the Soviet Republics of Kazakhstan and Uzbekistan. At the conclusion of the war, Soviet troops invading Manchuria captured many Unit 731 Japanese scientists and learned of their extensive human experimentation through captured documents and prisoner interrogations. Emboldened by the Japanese findings, Stalin put KGB (Committee of State Security) chief Lavrenty Beria in charge of a new biowarfare program. The production facility at Sverdlovsk was constructed using Japanese plans. After Stalin died in 1953, Beria was executed, and Nikita Khrushchev, the new Kremlin leader, transferred the biological warfare program to the Fifteenth Directorate of the Red Army. Colonel General Yefim Smirnov, a strong advocate of biological weapons who had been the chief of army medical services during the war, became the director.[67]

In 1956 Defense Minister Marshall Georgy Zhukov announced that the Soviet Union would be capable of deploying biological and chemical weapons in the next war. By 1960 numerous research facilities existed in the Soviet Union. Although the Soviet Union signed the 1972 BWC, it doubted US compliance, and subsequently expanded its program.[58,59,67] Various institutions under different ministries and production facilities were incorporated into an organization known as

Biopreparat to carry out offensive research, development, and production under the label of legitimate civil biotechnology research. Biopreparat conducted clandestine activities at 52 sites and employed more than 50,000 people. Production capacity for weaponized smallpox was 90 to 100 tons annually.[59]

The Soviet Union was an active participant in WHO's 1964 to 1979 smallpox eradication program. Soviet physicians participating in the program sent specimens to Soviet research facilities. For the Soviets, the program presented an opportunity not only to rid the world of naturally occurring smallpox, but also—reportedly—to obtain virulent strains of smallpox virus that could be used to develop biological weapons. WHO announced the eradication of smallpox in 1980, and the world rejoiced at this public health breakthrough. The bioweapon developers in the former Soviet Union had a more cynical reaction. Smallpox eradication would result in the termination of vaccination; eventually the world's population would again become vulnerable. It was this vulnerability that would inspire the former Soviet Union to develop smallpox as part of a strategic weapons system, with production of the virus on a massive scale and plans for delivery using intercontinental missiles.[59]

In addition to military biological weapons programs, the Soviets developed toxin weapons for use by Warsaw Pact intelligence services. An assassination using a biological weapon was executed in September 1978 when a Bulgarian secret service member attacked Georgi Markov, a Bulgarian exile living in London. A device concealed in an umbrella discharged a tiny pellet into the subcutaneous tissue of his leg. He died several days later. The pellet, which had been drilled to hold a toxic material, was found at autopsy. No toxin was identified, but ricin was postulated as the only toxin with the potency to kill with such a small dose.[68] Vladimir Kostov, a Bulgarian defector living in Paris, had been attacked in a similar manner a month earlier. He experienced fever and pain and bleeding at the wound site, yet had no further complications. After learning of Markov's death, he sought medical evaluation; radiographs disclosed a small metallic pellet in subcutaneous fat. The pellet was surgically removed. Kostov then tested positive for anti-ricin antibodies, supporting the probable use of ricin in these attacks.[23]

In October 1979 a Russian emigrant newspaper published in Frankfurt, Germany, reported a sketchy story of a mysterious anthrax epidemic in the Russian city of Sverdlovsk (now Yekaterinburg). The military reportedly took control of hospitals in Sverdlovsk to care for thousands of patients with a highly fatal form of anthrax. Soviet officials attributed the epidemic to cutaneous and gastrointestinal anthrax contracted from contaminated meat. However, US intelligence agencies suspected that the outbreak resulted from inhalational anthrax following a release of *B anthracis* spores from Compound 17, a Soviet military microbiology facility.[69–71] The Central Intelligence Agency sought the opinion of Matthew Meselson, a Harvard biologist who had been a strong proponent of the Nixon ban of the US biological warfare program. He initially doubted the Soviet weapon release hypothesis. Other observers reviewing the same evidence reached different conclusions, however, and satellite imagery from the late spring of 1979 showed a flurry of activity at and around the Sverdlovsk installation consistent with a massive decontamination effort. The incident generated enough concern within the Reagan administration and the Department of Defense (DoD) to increase military biopreparedness.

Debate of the incident raged for the next 12 years. Meselson testified before the US Senate that the burden of evidence supported the claim that the outbreak resulted from the Soviets' failure to keep *B anthracis*-infected animals out of the civilian meat supply. In 1992, after the fall of the Soviet Union, Meselson was allowed to take a team of scientists to review autopsy material and other evidence from the Sverdlovsk incident. The team's attempts to review hospital records of cases from the outbreak were unsuccessful because the KGB had confiscated the records. However, the team performed the following:

- acquired an administrative list of 68 of the deceased;
- obtained information from grave markers in a cemetery designated for the anthrax casualties;
- obtained epidemiological data by interviewing nine survivors and relatives and friends of 43 deceased; and
- determined that the cases occurred among people who had either lived or worked in a narrow zone southeast of Compound 17 during the first week of April 1979.

Archived weather reports at the city's airport disclosed that the wind direction on April 2, 1979, correlated with the geographic distribution of cases. Meselson and his team concluded that the outbreak resulted from the escape of aerosolized spores from the facility on April 2, 1979, with downwind transmission.[69] Furthermore, Russian pathologists who had conducted autopsies on 42 fatalities, and had courageously preserved tissue specimens and autopsy records at great personal risk, shared their findings with Meselson's team and published their results confirming inhalational anthrax,[72] described the Soviet cover-up of the outbreak, and postulated a release of spores from Compound 17.[71]

In 1992 Russian leader Boris Yeltsin admitted in private conversations with President George H Bush that the KGB and military had misrepresented the anthrax deaths. Subsequently, in a press release, Yeltsin admitted to the offensive program and the origin of the Sverdlovsk biological weapons accident. Additionally, retired Soviet general Andrey Mironyuk disclosed that safety filters had not been activated on the fateful morning in early April 1979, resulting in the escape of aerosolized *B anthracis* and the ensuing epidemic.[73] Soviet defectors, including Ken Alibek, first deputy chief of Biopreparat from 1988 to 1992, confirmed that not only was the Sverdlovsk epidemic caused by an accidental release of spores from a biological weapons production plant, but also that the Soviet biological warfare program had been massive.[59] In September 1992 Russia entered an agreement with the United States and the United Kingdom that acknowledged a biological weapons program inherited from the Soviet Union, committed to its termination, and agreed to onsite inspections. The United States assisted the Russian Federation and other former Soviet republics through the Nunn-Lugar Biological Threat Reduction Program (later called the Cooperative Biological Engagement Program) to:

- dismantle biological weapons research, development, and production infrastructure;
- secure dangerous pathogens into central reference laboratories;
- upgrade laboratory safety and security;
- enhance capacities for diagnosis, surveillance, and public health response; and
- engage scientists with biological weapons expertise in projects directed to modeling, medical countermeasure development, and other peaceful purposes.[74,75]

This led to the dismantlement or conversion of three large production facilities and dozens of institutes that supported the biological weapons program, the destruction of 150 tons of *B anthracis* weapons agent on Vozrozhdeniya Island, and unprecedented transparency at potential dual-use facilities that had previously been closed to foreigners.[76] However, in 1999 President Vladimir Putin, proposed the development of weapons based on new genetic technology. Although this directive was promptly dropped from publicly available documents, he retracted the 1992 disclosures of President Yeltsin.[77] The Russian government currently denies that the former Soviet offensive program had ever existed, claiming that it had only conducted defensive research.[58,77] According a 2013 US Department of State report, it is still unclear if the Russian Federation has completed the destruction or diversion of the offensive program to peaceful purposes, or if it continues to conduct activities inconsistent with the BWC.[78]

THE SPECIAL CASE OF IRAQ

The most ominous biological warfare threat that US military forces have faced came during Operations Desert Shield and Desert Storm in 1990 and 1991. Intelligence reports suggested that Iraq had developed and operated a biological weapons program during the 1980s. Coalition troops trained in protective gear were issued ciprofloxacin in theater for use as postexposure prophylaxis against an Iraqi anthrax attack. Before the hostilities, approximately 150,000 US troops received the Food and Drug Administration–licensed anthrax vaccine, and 8,000 received a botulinum toxoid vaccine approved by the Food and Drug Administration as an investigational new drug. Postwar inspections by the multinational UN Special Commission (UNSCOM) on Iraq were repeatedly confounded by Iraqi misinformation and obfuscation. After General Hussein Kamal defected in 1995, the Iraqi government disclosed that it had operated a robust biological weapons program at six major sites since the 1980s, contrary to its obligations as a state party to the BWC. The Iraqi program conducted basic research on *B anthracis*, rotavirus, camelpox virus, aflatoxin, botulinum toxins, mycotoxins, and an anticrop agent (wheat cover rust); and it tested several delivery systems including aerial spray tanks and drone aircraft. Furthermore, the Iraqi government had weaponized 6,000 L of *B anthracis* spores and 12,000 L of botulinum toxin in aerial bombs, rockets, and missile warheads before the 1991 Persian Gulf War (Table 1-2 and Table 1-3). Although these weapons were deployed, they were not used.[79,80] The reasons behind Saddam Hussein's decision not to use these weapons are unclear; perhaps

TABLE 1-2

BIOLOGICAL AGENTS PRODUCED BY IRAQ*

Agent	Produced (L)	Weaponized (L)
Botulinum	19,000	10,000
Bacillus anthracis	8,500	6,500
Aflatoxin	2,200	1,580

*Disclosed by the Iraq government in 1995.
L: liter

TABLE 1-3

DELIVERY SYSTEMS FOR BIOLOGICAL AGENTS DEVELOPED BY IRAQ*

Aerial Bombs		Missile Warheads	
Botulinum	100	Botulinum	13
Bacillus anthracis	50	*Bacillus anthracis*	10
Aflatoxin	16	Aflatoxin	2

*Disclosed by the Iraq government in 1995.

he was concerned about provoking massive retaliation. Alternately, decisive factors may have included the possible ineffectiveness of untested delivery and dispersal systems, the probable ineffectiveness of liquid slurries resulting from poor aerosolization, and the potential hazards to Iraqi troops, who lacked the protective equipment and training available to coalition forces.[81] The Iraqis claimed to have destroyed their biological arsenal immediately after the war but were unable to provide confirmatory evidence. A covert military research and development program continued for another 4 years, with the intent of resuming agent production and weapons manufacture after the end of UN sanctions. Infrastructure was preserved, and research on producing dried agent was conducted under the guise of biopesticide production at the Al Hakam Single Cell Protein Plant until its destruction by UNSCOM inspectors in 1996. Despite their obvious successes, the UNSCOM inspectors never received full cooperation from the Saddam Hussein regime, and were ejected from Iraq in 1998.

The Iraqi regime continued to promote an air of uncertainty after 1998 as to whether it had an active ongoing biological weapons program. Amy Smithson, in her very detailed account of the Iraqi biological weapons program and the UNSCOM inspections, suggests three possible reasons why Saddam Hussein may have wanted to maintain the perception that his biological weapons program was still active[82]:

1. To deter attacks by regional rivals, especially Iran;
2. To promote his image internally as a strong and unassailable leader and thus preserve his own internal stranglehold over Iraq; and
3. To maintain his own outsized vision of his ultimate dream and legacy.

Regardless of his strategic motives, the uncertainty about his biological weapons program ultimately contributed greatly to the Hussein government's fall and his own demise. The breakdown of the inspections, lack of firsthand information, misinformation provided by an informant (Rafid Ahmed Alwan al-Janabi, an Iraqi defector code named "Curveball" by the Central Intelligence Agency), and the 2001 anthrax mailings contributed to growing uncertainties, ambiguities, and apprehension, culminating in the 2002 US National Intelligence Estimate and assessments by the intelligence services of France, Germany, and the United Kingdom, that postulated a robust Iraqi biological weapons program.[83,84] International concern led to renewed inspections in 2002 under UN Security Council Resolution 1441. The Iraqi government failed to cooperate fully, and coalition forces invaded Iraq in 2003, believing at the time that Iraq's regime still posed a significant biological weapons threat. In 2005 the Iraq Survey Group (an international group composed of civilian and military members) concluded that the Iraqi military biological weapons program had been abandoned from 1995 through 1996 because the potential discovery of continued activity would risk severe political repercussions including the extension of UN sanctions. However, Saddam Hussein had perpetuated ambiguity regarding a possible program as a strategic deterrent against Iran.[85] The Iraqi Intelligence Service continued to investigate toxins as tools of assassination, concealed its program from UNSCOM inspectors after the 1991 Persian Gulf War, and reportedly conducted lethal human experimentation until 1994. Small-scale covert laboratories were maintained until 2003.[86]

OTHER NATIONAL PROGRAMS

South Africa is alleged to have operated a small-scale biological weapons program between 1981 and 1993, after becoming state-party to both the 1925 Geneva Convention (1960) and the BWC (1975). The South African biological weapons program, code-named Operation Coast, reportedly conducted research on *B anthracis*, *V cholerae*, ricin, botulinum toxin, and other agents, and intended to use genetic engineering to develop biological agents that would selectively target people of black African ancestry. Although Operation Coast acquired a collection of pathogens, it was not successful in developing large-scale delivery systems. *V cholerae* was reportedly used in 1989, but the attack failed because of the targeted water supply's chlorine content. After diplomatic interventions by the United States and Great Britain, the program was closed in 1993, coincident with the demise of the apartheid regime.[87–89]

V cholerae was allegedy used by Rhodesian forces with South African assistance during the civil war of the 1970s to contaminate rivers used as water

sources by rebel forces; these attacks are thought to have failed because of dilution. Rhodesian forces reportedly used *B anthracis* against livestock; the role of these attacks in an anthrax epizootic during 1979–1980 was investigated but could not be determined.[88]

Libya allegedly launched a clandestine biological weapons effort during the 1990s (while a state-party to the BWC), and sought assistance from Iraq, North Korea, and South Africa. However, in contrast to its chemical weapons program, the effort was limited to small-scale research, and according to one official never progressed beyond initial planning.[90] Colonel Muammar al-Qaddafi, an authoritarian dictator who ruled Libya for 42 years, formally renounced all weapons of mass destruction in 2003; inspectors from the United States and the United Kingdom found no evidence of an offensive biological weapons program.[90]

An unclassified 2013 US State Department report noted that North Korea may still consider the use of biological weapons as a military option, and that it is unclear if Iran is conducting activities prohibited by the BWC.[78]

The US Director of National Intelligence reported in an open US Senate hearing in 2013 that Syria (a signatory, but not a state-party to the BWC) maintains a biological weapons program capable of limited agent production; and although Syria is not known to have loaded biological agents in effective delivery systems, it possesses conventional and chemical weapons devices that could be adapted to launch biological attacks.[91,92] In the context of the ongoing Syrian civil war in 2014, there are concerns regarding potential deployment[93] and that further disintegration of the Assad regime could enable Al Qaeda and Hezbollah to seize Syrian unconventional weapons.[94]

Some 20 nations are thought to have engaged in offensive biological weapons efforts. The total number of nations and the extent of their efforts are difficult to establish because several have engaged in research and development, but not taken their efforts to testing, deployment, and use. Although the list of states appears to be down from the 20 or so that were thought to have biological weapons programs in the assessments in the 1980 and 1990s, several states including North Korea, Syria, and Iran are still thought to have biological weapons programs.[54(p68)]

BIOCRIMES

Biocrime is the malevolent use of biological agents when the perpetrator's motivation is personal, as opposed to a broader ideological, political, or religious objective. Although biocrimes constitute only a small fraction of criminal assaults and are usually unsuccessful,[95] a well-executed attempt may be deadly; the resulting disease may pose clinical and forensic challenges. Biocrimes have generally been more successful than bioterrorist attacks; 8 of 66 biocrimes reviewed by Tucker produced 29 deaths and 31 injuries.[96]

Perpetrators with scientific or medical expertise or those who have recruited trained accomplices typically attempt biocrimes. Criminals without a technical background have successfully extracted ricin from castor beans but have generally been unable to obtain or produce other agents. In a review of 14 episodes in which agent was used, biological agents were usually obtained from a legitimate source or stolen; the perpetrators produced agent in only two cases.[21,95] Preferred agents have been bacteria and toxins (eg, ricin). Food contamination has been preferred over direct injection or topical application as a means of attack.

One of the most striking examples of foodborne biocrime occurred in Japan between 1964 and 1966. Dr Mitsuru Suzuki allegedly contaminated food items, medications, barium contrast, and a tongue depressor with *Salmonella typhi* and agents of dysentery on numerous occasions resulting in more than 120 cases and four deaths.[23] A variation on the Suzuki crime occurred in 1996 when Diane Thompson, a hospital microbiologist, deliberately infected 12 coworkers with *Shigella dysenteriae*. She sent an email to her coworkers inviting them to eat pastries she had left in the laboratory break room. Eight of the 12 casualties and an uneaten muffin tested positive for *S dysenteriae* type 2, identical to the laboratory's stock strain by pulsed-field electrophoresis.[97] Police learned that her boyfriend had previously suffered similar symptoms and had been hospitalized at the same facility, and that Thompson had falsified his laboratory test results. Thompson was sentenced to 20 years in prison.[23]

Murders by direct injection included the use of diphtheria toxin in Russia in 1910. The director of a Norwegian nursing home was convicted in 1983 of murdering 22 patients by injecting a curare derivative. There have been at least four murder attempts by injecting victims with human immunodeficiency virus-infected blood.[23]

Numerous and highly varied biocrimes have been reported; only several representative examples are included in this chapter. The works of Carus,[23] Leitenberg,[21] and Tucker[96] provide comprehensive descriptions and analysis.

BIOLOGICAL TERRORISM

Bioterrorism is the use of biological agents by an individual or group not acting as official agents of a government to achieve a political or ideological objective. Bioterrorist incidents increased markedly after 1985, with two peaks in 1998 and 2001. The 1998 peak followed publicity of the anthrax threat posed by Larry Wayne Harris; the 2001 peak followed the September through October anthrax mailings. Successfully executed attacks have been few but high in impact; the 1984 Rajneeshee Salmonella attack resulted in 751 cases of infection; the 2001 anthrax mailings resulted in 22 cases of infection, five deaths, and approximately 10,000 individuals being offered postexposure prophylaxis. The vast majority of incidents (at least 98% during 2000–2002) have been hoaxes, which have nonetheless produced considerable social disruption.[98,99]

The first large-scale bioterrorism attack in the United States occurred in 1984. In the 1960s an Indian guru named Bhagwan Shree Rajneesh founded the Rajneeshee cult. Rajneesh succeeded in attracting followers from the upper middle class and collecting significant donations and proceeds from book and tape sales. Rajneesh acquired the Big Muddy Ranch near The Dalles, Oregon, and built a community for his followers named Rajneeshpuram, which became an incorporated community. Within a few years, the Rajneeshees came into conflict with the local population regarding development and land use. The Rajneeshees attempted to gain control of the Wasco County government by bringing in thousands of homeless people from cities around the country, counting on their votes in the upcoming elections. The Rajneeshees also plotted to sicken the local population to prevent them from voting.[21]

Two Wasco County commissioners visiting Rajneeshpuram on August 29, 1984, were given drinking water contaminated with *Salmonella typhimurium*; both became ill and one was hospitalized. In trial runs in the months leading up to the November 1984 elections, several attempts at environmental, public water, and supermarket food contamination were unsuccessful. In September, Rajneeshees began contaminating food at local restaurants by pouring slurries of *S typhimurium* into salad bars, salad dressing, and coffee creamers at 10 restaurants. This attack caused 751 cases of enteritis and at least 45 hospitalizations.[23,100]

In 1995 in Japan, the Aum Shinrikyo cult released sarin gas in the Tokyo subway system, resulting in 12 deaths and thousands seeking emergency care. The cult, founded by Shoko Asahara, had amassed approximately 10,000 members and $300 million in financial assets. Aum Shinrikyo mimicked the organization of the Japanese government with "ministries and departments." Seiichi Endo, who headed "health and welfare," had worked in genetic engineering at Kyoto University's viral research center. Hideo Murai, who headed "science and technology," had an advanced degree in astrophysics and had worked in research and development for Kobe Steel Corporation. Endo attempted to derive botulinum toxin from environmental isolates of *Clostridium botulinum* at the cult's Mount Fuji property. A production facility was built and horses were stabled for developing a horse serum antitoxin. It is uncertain whether Endo successfully produced potent botulinum toxin.[23]

In 1993 Aum Shinrikyo built a new research facility on the eighth floor of an office building owned by the cult in eastern Tokyo. The cult grew *B anthracis* and installed a large industrial sprayer for dissemination. The cult is also believed to have worked with *C burnetii* and poisonous mushrooms, and it sent a team to Zaire in the midst of an Ebola epidemic to acquire the Ebola virus. According to press accounts from 1990 to 1995, the cult attempted to use aerosolized biological agents against nine targets. Three attacks were attempted with *B anthracis* and six with botulinum toxin. In April 1990 the cult equipped three vehicles with sprayers containing botulinum toxin targeting Japan's parliamentary Diet Building in central Tokyo, the city of Yokahama, Yosuka US Navy Base, and Nairta International Airport. In June 1993 the cult targeted the wedding of Japan's crown prince by spraying botulinum toxin from a vehicle in downtown Tokyo. Later that month, the cult spread *B anthracis* using the roof-mounted sprayer on its eight-story building. In July 1993 the cult targeted the Diet in central Tokyo again by using a truck spraying *B anthracis*, and later that month it targeted the Imperial Palace in Tokyo. On March 15, 1995, the cult planted three briefcases designed to release botulinum toxin in the Tokyo subway. Explanations for the cult's failure include the possible use of a nontoxin-producing (or low yield) strain of *C botulinum*, use of a low-virulence veterinary vaccine strain of *B anthracis*, ineffective spraying equipment, and perhaps subversion on the part of some cult members who were reluctant to execute the planned operation.[19] Ultimately, Aum Shinrikyo gave up on its biological weapons and released sarin in the Tokyo subway on March 20, 1995.[23]

Meanwhile in the United States, two members of the Minnesota Patriots Council, an antigovernment extremist group, were arrested for producing ricin and planning to attack federal agents by contaminating doorknobs. Larry Wayne Harris, a clinical

microbiologist with ties to racist groups, was arrested in 1995 for using fraudulent information to obtain a culture of *Y pestis* from the American Type Culture Collection. He was arrested a second time in 1998 after making threatening remarks to US federal officials and violating his parole. Harris had constructed a covert laboratory in Nevada and was conducting experiments with the Sterne strain of *B anthracis*, a nonencapsulated but toxigenic live attenuated veterinary vaccine, and he threatened to attack Las Vegas with *B anthracis*.[68] His case led to the establishment of the Select Agent Program (42 CFR Part 73, Possession, Use, and Transfer of Select Agents and Toxins) that included the development of stringent regulations for the procurement and shipping of select microbes.

On October 4, 2001, just 3 weeks after the September 11th attacks on the World Trade Center and the Pentagon had made the nation acutely aware of its vulnerability to international terrorism, health officials in Florida reported a case of inhalational anthrax. During the first week of September, American Media, Inc, received a letter addressed to Jennifer Lopez containing a fan letter and a "powdery substance." The letter was passed among its employees, including Robert Stevens. Retrospectively, investigators would consider not this letter, but perhaps a subsequent letter, as the source of his infection.[101]

Stevens was admitted to a Palm Beach, Florida, hospital with high fever and disorientation on October 2, 2001. By October 5, he was dead from inhalational anthrax, the first such case in the United States in more than 20 years.

Soon afterward anthrax mailings were received at civilian news media operations in New York City and in the Hart Senate Office Building in Washington, DC.

At least five (four recovered) letters containing *B anthracis* spores had been mailed on September 18, 2001, and October 9, 2001. Twenty-two people contracted anthrax, with 11 inhalational cases resulting in five deaths. Thirty-five postal facilities and commercial mailrooms were contaminated. Screening and postexposure prophylaxis disrupted operations at the Hart US Senate Office Building. Decontamination of postal facilities cost more than $1.2 billion and resulted in the closure of heavily contaminated facilities in Washington, DC (October 2001–December 2003), and Trenton, New Jersey (October 2001–March 2005).[102] More than $27 million was spent on decontaminating Capitol Hill facilities.[102] Public alarm was compounded by numerous "white powder" hoaxes.

Farsighted emergency planning and training, in addition to the integration of federal and local medical, public health, and law enforcement agencies in New York City and other cities, enabled an unprecedented public health response. The Laboratory Response Network and military laboratories such as USAMRIID processed more than 125,000 clinical specimens and 1 million environmental samples. USAMRIID ran more than 260,000 assays on more than 30,000 samples in 9 months. Prophylaxis supplied from the national stockpile was offered to nearly 10,000 individuals at risk. No cases were found among prophylaxis recipients.[103,104] Treatment guidelines advocating multidrug antibiotic combinations and aggressive intensive care were disseminated,[105] and the case fatality rate for inhalational anthrax—historically exceeding 90%—reduced to 45%.[106,107]

The attacks provoked an unprecedented criminal investigation that coupled traditional law enforcement with the development and validation of novel emerging genetic sequencing techniques. The Federal Bureau of Investigation (FBI) special agents and US Postal Service Inspectors conducted the investigation for 7 years, and 29 government, academic, and commercial laboratories supported it. Investigators conducted more than 10,000 witness interviews on six continents and 80 searches, and they also collected more than 6,000 items of potential evidence and 5,730 environmental samples from 60 locations both within the United States and in foreign countries, with the cooperation of the respective host nation governments.[102]

US Attorney General John Aschroft named Dr Steven J Hatfill, a USAMRIID scientist between 1997 and 1999, a "person of interest" during a television interview in 2002. Dr Hatfill vehemently denied involvement, and sued the federal government, claiming that law enforcement officials had leaked information to the media in violation of the Privacy Act, and had ruined his reputation and career. The FBI exonerated him in 2008, and he received $5.82 million in restitution.[102,108,109]

Forensic analysis was confounded by the highly conserved *B anthracis* genome, which features more than 99.99% nucleotide sequence identity among the most genetically divergent strains. Investigators went beyond the contemporary standard of genetic typing by sequencing small DNA segments to advance the technique of whole genome sequencing. Comparison of the whole genomes of the index case isolate and a reference Ames strain disclosed that they were essentially identical, and it could not pinpoint the origin of the letter contents. However, a breakthrough followed the observation of four phenotypic colony morphology variants constituting less than 1% of colonies cultured from spore samples taken from three of the anthrax letters. Each colony morphology variant was associated with a distinct mutation restricted to four genetic loci. These mutations were absent in environmental

isolates taken during the investigation.[110] Specimens were obtained from every culture of *B anthracis* Ames strain (1,071 samples) from all 15 US and three foreign laboratories known to possess it. One or more of the mutants was detected in 71 of 947 samples that could be evaluated; all four mutants were present in eight samples. The probability of samples to contain all four mutants was calculated to be 0.4383×10^{-6} or 0.0004 samples in the 947 sample collection, if the samples were unrelated; these eight samples consisted of a specimen from RMR-1029, a flask containing a liquid spore preparation in the laboratory of anthrax researcher Dr Bruce E Ivins at USAMRIID, and seven specimens from another laboratory that were descendents of RMR-1029.[111,112]

The FBI concluded that Dr Ivins was the sole perpetrator based on the following:

- the genetic analysis results;
- inconsistencies during interviews;
- erratic conduct that included irregular laboratory hours before each mailing and an unauthorized and unreported decontamination of his office and laboratory during the investigation;
- deteriorating behavior as the investigation progressed; and
- exclusion of other individuals with access to RMR-1029 and its descendants.

The purported motive was to ensure continued support for the anthrax vaccine research in which Dr Ivins was personally heavily invested and was under criticism from multiple sectors. The US Attorney's Office for the District of Columbia prepared an indictment charging him with Use of a Weapon of Mass Destruction, in violation of Title 18, United States Code, Section 2332a, and related charges. Dr Ivins, aware of the indictment, took an overdose of over-the-counter medications and died on July 29, 2008.[102]

Lingering doubts were expressed during a plenary session at the 2009 American Society for Microbiology Biodefense and Emerging Diseases Research Conference.[113] Evidence was considered circumstantial. No evidence of *B anthracis* contamination was found in Dr Ivin's home or vehicles. Unexplained aspects of the case included the contamination of the September 18 mailing with a *B subtilis* strain that could not be traced to USAMRIID and the use of dry spore preparations (the production of which is prohibited in the US biodefense program), for which there was no direct evidence within USAMRIID. A National Academy of Sciences review concluded that the genetic typing results were consistent with—but not definitive proof of—the derivation of the letter isolates from RMR-1029. Although generally supportive of the FBI's efforts, the reviewers criticized the FBI's statistical methods and stated that an alternative source could not be excluded because of possible sharing and mixing of samples among laboratories, and because the possibility of identical mutations arising through parallel evolution independently in unrelated cultures had not—in their opinion—been adequately explored.[112] Abnormally high concentrations of silicon[114] and tin existed in the spores that were absent in spores from RMR-1029; this raised controversies regarding potential production at the Dugway Proving Ground or at a civilian contractor laboratory, where work with silicon and surrogate spores had previously been done.[115] Finally, Department of Justice lawyers used the argument that Dr Ivin's lab had no equipment to produce dry spore preparations to defend the government against a wrongful death lawsuit filed by Robert Stevens' widow.[116]

However, the investigation spurred the advancement of whole genome sequencing, accelerating the time required to sequence a bacterial genome from 4 months to several days,[117] and advanced the emerging science of microbial forensics. The investigation raised issues regarding laboratory programs for physical security, personal reliability, and mental health screening that—while not directly incriminating Dr Ivins—underscored the importance of re-evaluating laboratory security measures and the value of robust employee occupational health programs to screen and monitor the mental health of researchers working with highly virulent pathogens. These issues were addressed by strengthening the federal regulations that direct CDC oversight of research on dangerous pathogens (see discussion of the Federal Experts on Security Advisory Panel in Toward Pan-hazard Preparedness).[118,119]

The threat of bioterrorism did not end with the US anthrax experience. Al Qaeda initiated a biological weapons program in Afghanistan before the overthrow of the Taliban regime. Investigations after the US military intervention of 2001 uncovered two Al Qaeda laboratories for biological weapons development, supplied with commercially acquired microbiology equipment and staffed by trained personnel. Fortunately, a deployable weapon had not been constructed.[120] US forces operating in northern Iraq in 2003 seized a camp linked to Al Qaeda reportedly containing instructions and equipment for ricin extraction.[121]

During the period that followed the US anthrax attacks, ricin became the bioweapon of choice for a number of misanthropes intent on nefarious use of biological agents, perhaps because of its relative ease of access. The castor beans (ricin source) are available worldwide because the oil is extracted for lubricant in

many countries. The toxin extraction techniques have been published in many forums to include many anarchist and terrorist websites. Examples are provided of confirmed cases, but many more incidents have occurred worldwide, and most have proven to be hoaxes.

In January 2003 British authorities uncovered the Wood Green ricin plot. A police raid on a London apartment yielded a copy of a protocol for ricin production, toxin source materials (castor beans), and a suitable solvent (acetone) for its extraction. Although tests for ricin were negative,[122] one of the tenants, an Al Qaeda-trained operative, was convicted of plotting a ricin attack. He had planned to contaminate handrails in the railway system connecting London and Heathrow Airport.[123] In March 2003 two flasks containing ricin were discovered in a railway station in Paris.[124]

In 2003 US Postal Service employees discovered two letters directed to the US Department of Transportation containing vials of ricin. The first letter was found on October 15, 2003, at the mail sorting center in Greenville, South Carolina.[125] The second was discovered at the White House mail processing facility in Washington, DC. Both letters were from an antagonist who identified himself as "Fallen Angel" and was angry about the Department of Transportation's new limitations being placed on truck drivers' daily work hours.[126] In February 2004 ricin was found in the sorting machine of Senate Majority Leader Bill Frist's office in the Congressional Office Building. No evidence was ever found linking the Fallen Angel and Frist cases and perpetrators are still at large. On June 23, 2004, Michael Crooker, a resident of the Boston suburb of Agawam, Massachusetts, had his house searched by law enforcement officials after attempting to mail a firearm. Agents discovered a weapons lab that contained castor and abrus seeds (sources of ricin and abrin toxins, repectively) as well as the materials needed for toxin extraction. Crooker sent a letter to the prosecuting attorney threatening to cripple the US Postal System by sending toxin-laden letters through the mail. He also notified local news journalists that he would provide toxins to felons he had met in prison who had previously engaged in terrorist activities. He pled guilty to possession of ricin and threatening a government official and was sentenced in June 2011.[127] In February 2008 Roger Bergendorff, an anarchist living in an extended stay hotel in Las Vegas, Nevada, developed a mysterious illness that puzzled his healthcare providers. He was hospitalized and while investigating the cause of his illness, officials discovered evidence of a ricin extraction operation in his room. He and his cousin were both eventually convicted of charges related to ricin production. The specifics of intended use—if known—have not been disclosed.[128] In March 2011 four men who were members of a militia organization began having clandestine meetings in which they allegedly planned numerous criminal activities to include acquisition of illegal weapons, manufacture of toxic agents, theft, and assassination. During these meetings they allegedly discussed use of weapons to include biologic agents to attack government facilities and government employees to include law enforcement officials. One of their plans included producing 10 pounds of ricin and dispersing it from a moving vehicle in the Atlanta area. An FBI informant alerted authorities and the operation was disrupted without incident in November 2011.[129]

Attacks against government officials resumed after a nearly 10-year hiatus with the discovery of an envelope testing positive for ricin intercepted at the US Capitol's mail facility in April 2013. The letter was addressed to Senator Roger Wicker, and a day later an envelope addressed to President Obama was discovered that also contained ricin. A third letter containing ricin was mailed to the Lee County Mississippi Court Judge Sadie Holland. Within a few weeks the FBI arrested Everett Dutschke for producing a toxin weapon and using the mail to threaten President Barack Obama, Senator Wicker, and Judge Holland. These mailings appear to be acts of reprisal in the settlement of personal grudge(s).[130] Less than 2 months later, in May 2013 three letters intended for New York Mayor Michael Bloomberg were intercepted containing a suspicious oily substance that turned out to contain ricin. Similar letters were also mailed to President Obama, according to a Secret Service press release. Gun control opponents purportedly sent the letters, and Shannon Richardson notified the FBI that her estranged husband was responsible for the mailings. When the allegations failed to withstand police scrutiny she was arrested, and received an 18-year prison sentence, having falsely implicated her husband.[131] Despite numerous ricin mailings by many diverse individuals, the mail delivery of ricin toxin has been ineffective as an instrument of harm or assassination—these mailings appear to have little impact beyond their psychological "scare" effect. Although ricin is a toxin of very high lethal potency, its effectiveness is limited by the delivery method. No illness or significant environmental contamination has resulted from any of the ricin mailings.

Many of the bioterrorist incidents have been small scale, not well perpetrated, and not particularly successful in terms of mortality and morbidity. Still, it is clear that several terrorist groups aspire to use biological weapons. For example, Al Qaeda radical cleric Anwar al-Awlaki in an article stated that "the killing of women and children and the use of chemical and biological weapons in addition to bombings and gun attacks" is acceptable and even encouraged.[54]

In Inspire, an online Al Qaeda magazine, the authors called for "chemists and microbiologists" to develop weapons and attack the West. These programs continue to be aspirational, rather than well-established developmental efforts. However, with the proliferation and industrialization of biotechnology described previously, the threat of bioterrorism continues to increase.[54(p60)]

SOLUTIONS: TOWARD PAN-HAZARD PREPAREDNESS

Disarmament: The Biological Weapons Convention

In July 1969 Great Britain issued a statement to the UN Conference of the Committee on Disarmament calling for the prohibition of the development, production, and stockpiling of bacteriological and toxin weapons. In September 1969 (the same year) the Soviet Union unexpectedly recommended a disarmament convention to the UN General Assembly. In November 1969 WHO issued a report on biological weapons, after an earlier report by the 18-nation Committee on Disarmament, describing the unpredictable nature, lack of control, and other attendant risks of biological weapons use. The United Nations then developed the 1972 Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction (1972 BWC), which prohibited any malicious research, production, or possession of biological agents. Among the 103 initial cosignatory nations, agreement was reached to "never develop, produce, stockpile, or otherwise acquire or retain microbiological agents or toxins, whatever their origin or method of production, of types and in quantities that have no justification for prophylactic, protective or other peaceful purposes; and weapons, equipment or means of delivery designed to use such agents or toxins for hostile purposes or in armed conflict."[132] The United States ratified both the 1925 Geneva Convention and the BWC in 1975. Signatory states suspecting others of treaty violations may file a complaint with the UN Security Council, which, in turn, may order an investigation. However, mandatory measures for verification and enforcement are lacking; numerous attempts to formulate such measures have been unsuccessful because of political, security, and proprietary issues.[21]

Since the BWC entered into force in 1975, seven review conferences have taken place; these "RevCons" (as they are called) constitute the only decision-making forums for the BWC and are held every 5 years in Geneva. RevCons are 3-week international meetings that allow member nations to reinforce the norm against the prohibition of biological weapons, discuss international collaboration on biotechnological issues, assess the continued relevance of the BWC given changes in biotechnology, and make proposals for revitalizing the BWC. Unfortunately, RevCons have not produced many tangible results and have demonstrated an inability to deal with difficult issues. The most noteworthy accomplishment was development of confidence-building measures for annual reporting by member state parties. Only 70 or so of the 170 member nations actually submit annual reports on their activities. On questions such as the relationship of the Sverdlovsk anthrax epidemic to the Soviet biological weapons program, the Iraqi weapons program, and the smallpox retention versus destruction issue, the BWC has remained unengaged.

Several RevCons have dealt directly with the potential for developing a verification protocol. The 1991, 1996, and 2001 RevCons saw the establishment of the Ad Hoc Group, the progress made in the Review Conference Final Declaration, and the disaster of the United States walking out of the RevCon,[54(p117)] respectively. After the 2001 RevCon the BWC saw a tumultuous period where its future was questioned. The "success" of the 2006 RevCon served to reenergize the BWC. The key outcomes were the agreement concerning the importance of the BWC forum and the development of an intersessional process that would include annual member state nations and experts meetings to discuss topical issues. However, neither of these two new annual meetings allows for decision-making.

The lead-up to the 2011 RevCon was anticipated by participating nation-states.[54(p119)] The United States had released a national strategy for countering biological threats at the 2009 meeting of state parties. Several pre-BWC conferences were held in which it appeared the international community was moving toward tangible outcomes in the 2011 RevCon. The president of the 2011 meeting, Paul van den Ijssel from The Netherlands, had declared the mantra would be "ambitious realism."[54(p122)] Unfortunately, it failed to live up to expectations. One review of the RevCon states, "The December 2011 review conference of the Biological Weapons Convention (BWC) demonstrated the danger of the bioweapons ban drifting into irrelevance. Standstill was the motto of the meeting. Only incremental improvements on some procedural issues were achieved."[54(p120)] Even modest enhancements, such as expanding the implementation support unit's three-person organization, were not approved.

In examining the BWC's future, several tensions arise because it is a state-to-state treaty, yet many of the current biological threats deal with nonstate issues

such as bioterrorism, biocrimes, and misuse of the life sciences. Although member nations allow for discussing these issues within the BWC, few have demonstrated the desire to make these more topical issues the focus of future BWC negotiations, although states-parties are obligated under article IV to prohibit and prevent proscribed activities within their borders. Several other articles of the BWC also create tensions. For example, article I establishes the norm against biological weapons, yet provides no ability to enforce the convention. Articles III and X call for not transferring, assisting, inducing, acquiring, and retaining biological weapons, whereas article X encourages the peaceful exchanges of biological science and technology. Although the words do not conflict, the interpretation between developed and developing nations varies greatly.

Another area of contention concerns the perennial issue of verification. The US position remains as it has since 2001 that verification of the BWC is not possible. Instead, the United States supports adherence to a policy of compliance that begins with national implementation including ensuring all nations have appropriate national laws, regulations, and policies that support the BWC, as stipulated in article IV. The US position on verification also rests on the assertion that articles V and VI that call for bilateral and multilateral consultation and the potential for bringing concerns to the UN Security Council, respectively, provide sufficient opportunities for voicing concerns about compliance. Two other issues that feature prominently in the BWC debate are continued concerns about its relevance given the pace of biotechnological enhancements and the lack of universal adherence to it. On the first issue, members continue to profess that the BWC remains relevant despite exponential changes in biotechnology. With respect to universal adherence, the BWC continues to be undersubscribed as compared to other treaties dealing with weapons of mass destruction issues, in particular the Nuclear Non-Proliferation Treaty and the Chemical Weapons Convention. The BWC has 170 member nations, whereas the Nuclear Non-Proliferation Treaty and the Chemical Weapons Convention have 189 and 188 member nations, respectively.

Finally, only one allegation has been formally registered under the BWC: in June 1997 Cuba accused the United States of a biological attack with a crop pest insect, *Thrips palmi*. The allegations were unsubstantiated in a BWC consultation that concluded in December 1997.[21] Other attempts at biological arms control have been conducted outside of the context of the BWC; for example, inspections and sanctions against Iraq from 1991 to 1998 and 2002 to 2003 were accomplished under separate UN Security Council Resolutions, 681 and 1441, respectively.

Smallpox Preparedness

CDC launched a comprehensive smallpox preparedness program in 2002 because of the potential use of variola as a biological weapons agent. WHO, the United Kingdom, Germany, and other WHO member states initiated similar programs including vaccine stockpiles. The US program integrated community, regional, state, and federal healthcare and public health organizations and featured logistical preparation; training and education; risk communication; surveillance; and local preparations for mass vaccination, isolation, quarantine, active surveillance, and humane treatment of patients in designated facilities. A strategy was adopted based on preexposure vaccination of carefully screened and trained members of first-response teams, epidemiological response teams, clinical teams at designated facilities, and military personnel set to deploy into the theaters of war.[133] More than 400,000 selected military personnel and 38,000 civilian emergency responders and healthcare workers in designated smallpox response teams were vaccinated. Contracts for the production of a new cell culture-derived vaccine were awarded in 2000; the Strategic National Stockpile has sufficient cell culture-derived vaccine for the entire US population, a replication-deficient vaccinia (Modified Vaccinia Ankara) for use in immunocompromised individuals, and vaccinia immune globulin to treat vaccine complications. In addition, the US government supported the development of new smallpox antiviral therapeutic candidates and funded animal model development to enable efficacy testing of medical countermeasure candidates.

The disposition of the remaining WHO-authorized variola virus stocks, held in two secure WHO Collaborating Centers at CDC in the United States and at VECTOR in Koltsovo, Novosibirsk, Russia, was debated at the WHO 64th World Health Assembly in 2011. Two camps emerged, the destructionists and retentionists, and each made arguments to support their positions. In the end, the World Health Assembly remained committed to its previous position calling for the destruction of the viral stocks as a long-term goal, but agreed to their retention until the completion of research leading to two antiviral drugs with different mechanisms of action, a safer and effective vaccine, a rapid and accurate diagnostic kit, and the refinement of nonhuman primate animal models. The issue was also revisited at the 67th World Health Assembly in 2014. The risks posed by recombinant technology were also addressed; a private company in the United States that had inserted 63 nucleotides from the variola genome into an attenuated but transmissible orthopox virus to develop a positive control for a diagnostic test

would be asked to destroy its reagent and to report its destruction to WHO.[134] This underscored the need to re-evaluate and publicize WHO guidance regarding the use of variola genetic sequences in recombinant technology.

Dual Use Research of Concern

In addition to the threats posed by the deliberate release of biological agents, there has been increasing recognition of the potential risks posed by legitimate scientific research for benevolent medical purposes that includes the characterization of, and development of medical countermeasures against, highly pathogenic microbes. Risks include laboratory accidents resulting in pathogen release, laboratory acquired infections (some of which may be communicable to the community), unanticipated results of experiments resulting in increased microbial virulence or transmissibility, and the deliberate misuse of knowledge generated by legitimate scientific research for biological weapons proliferation. Dual use research of concern (DURC) has been identified as biological research with legitimate scientific purpose that may be misused to pose a biologic threat to public health and/or national security. Examples include, but are not limited to, the following:

- The genetic modification of mousepox virus to express both an ovarian protein and the immunomodulator interleukin-4 to induce sterility in mice for pest control, reported in 2001. Immunomodulator interleukin-4 was intended to enhance immune responses to the ovarian protein. However, the vaccine candidate was lethal in small-animal testing; immunomodulator interleukin-4 had the unanticipated effect of immune suppression, resulting in a highly virulent mousepox virus.[135]
- The in vitro synthesis of wild-strain poliovirus type 1 by using synthetic DNA encoding the poliovirus genome (with minor mutations as genetic markers) in a cell-free extract by researchers at the State University of New York at Stony Brook in 2002. The researchers noted that the knowledge that polioviruses can be synthesized using chemical methods and reintroduced through bioterrorism may inform the closing strategies of WHO's polio eradication campaign.[136,137] It was later explained that they had hoped to deliver a "wake-up call" regarding the possible misuse of viral synthesis for bioterrorism; that WHO's polio eradication campaign may be futile because of either possible bioterrorism using synthetic virus, laboratory accidents, or live attenuated oral polio vaccine and circulating oral polio vaccine-derived virus-related disease; and that control may be a more attainable outcome.[138] Aside from risking an accidental reintroduction to the local community (after the elimination of circulating wild-strain polioviruses from the western hemisphere), the study raised questions regarding its scientific value,[139] whether demonstrating technical capabilities to deliver warnings constitutes a legitimate scientific purpose, and whether the synthesis of a wild-strain poliovirus, which is otherwise available to researchers, served any benevolent medical purpose.
- The reconstruction of the 1918 H1N1 influenza A pandemic virus,[140] reported in 2005. This enabled characterization of a virulent pathogen that—in contrast to poliovirus—was otherwise not available for study. This enabled insights into pathogenesis, and potentially the identification of virulence factors and drug targets that could be relevant to counter future pandemic strains.[141] Using appropriate biosafety and biosecurity measures minimized risks to the public.
- The generation of a mutant of the highly pathogenic avian influenza A virus H5N1 (HPAI H5N1) with enhanced transmissibility between mammalian hosts (ferrets) that was as contagious as seasonal influenza viruses and retained the virulence of the wild strain[142,143] (55%–60% mortality in humans) by researchers at Erasmus University (although it was later reported that the mutant was attenuated and not as communicable as originally claimed), reported during the autumn of 2011. Concurrently, researchers at the University of Wisconsin developed a recombinant 2009 pandemic H1N1 virus expressing H5 hemagglutinin receptor binding proteins that was transmissible between ferrets. These announcements stunned many in the scientific community and the general public as risking a pandemic catastrophe following a laboratory accident or intentional release. Policy makers became concerned that the publication of these studies would support biological weapons proliferation by providing information that could be used to produce highly communicable and lethal influenza viruses.

Within 4 months of the publication of poliovirus synthesis, the Center for Strategic and International Studies and the National Academy of Sciences held a workshop on scientific openness and national security that involved a wide stakeholder community from government, academia, and scientific editorial communities that generated voluntary guidelines for ensuring the publication of new knowledge while safeguarding information that may pose security risks. Issues raised by DURC led to the foundation of the National Science Advisory Board for Biodefense (NSABB) in 2004. NSABB is a federal advisory committee within the Office of Science Policy in the National Institutes of Health (NIH) that provides advice, guidance, and leadership regarding biosecurity oversight of dual use research, defined as biological research with legitimate scientific purpose that may be misused to pose a biological threat to public health and/or national security. NSABB is chartered to recommend strategies and guidance for enhancing personnel reliability among individuals with access to biological select agents and toxins; provide recommendations on the development of programs for outreach, education, and training in dual use research issues for scientists, laboratory workers, students, and trainees in relevant disciplines; advise on policies governing publication, public communication, and dissemination of dual use research methodologies and results; recommend strategies for fostering international engagement on dual use biological research issues; advise on the development, utilization, and promotion of codes of conduct to interdisciplinary life scientists and relevant professional groups; advise on policies regarding the conduct, communication, and oversight of dual use research and results, as requested; advise on the Federal Select Agent Program, as requested; and address any other issues as directed by the Secretary of Health and Human Services. NSABB concerns include knowledge, products, or technologies that may:

- enhance the harmful consequences of a biological agent or toxin;
- disrupt the immunity or the effectiveness of an immunization without clinical and/or agricultural justification;
- confer to a biological agent or toxin, resistance to clinically and/or agriculturally useful prophylactic or therapeutic interventions or facilitate their ability to evade detection methodologies;
- increase the stability, transmissibility, or the ability to disseminate a biological agent or toxin;
- alter the host range or tropism of a biological agent or toxin;
- enhance the susceptibility of a host population; and
- generate a novel pathogenic agent or toxin or reconstitute an eradicated or extinct biological agent.

Examples of initiatives coordinated through NSABB include Department of Health and Human Services (DHHS) guidelines for synthetic biology[144] and guidance for providers of double-stranded DNA to screen procurement orders.[145]

In December 2011 NSABB reviewed manuscripts of the Erasmus University and University of Wisconsin studies on enhanced transmission of HPAI H5N1 that were being prepared for publication and made the unprecedented, nonbinding recommendation to redact methods and experimental details.[146] In addition, the influenza research community voluntarily invoked a moratorium on gain-of-function research using HPAI H5N1.

NSABB members asserted that their recommendation was an exceptional and adaptive response to a special case—a situation generated by the life sciences, biodefense, and general public communities being caught off-guard—and having limited awareness of the research until the manuscripts were being prepared (even though NIH had funded both projects), they reasoned that:

- in the future, the value of conducting and supporting specific dual use research projects should be carefully considered a priori by a wide stakeholder community including experts in life sciences, biosecurity, and members of the general public; and
- decisions to publish results should follow the principle of "do no harm," with the best interest of public health in mind.[147]

However, supporters of the research and its publication argued that:

- medical science must address the most virulent pathogens to be valuable;
- new knowledge of determinants of transmissibility may be useful to predict the likelihood of an epi- or enzootic virus being capable of a "species jump" to humans and consequent person-to-person transmission;
- the mutants afforded an opportunity to test vaccine and therapeutic candidates against potential future emerging viruses;

- methods used in the studies are already well-known in the scientific community;
- persons with malicious intent could use simpler means to inflict disease and injury; and
- redacting the manuscripts constituted censorship, thus violating long-standing principles of academic freedom.[148–151]

As the debate raged,[152–156] WHO concluded that such research and its publication is in public health's best interest and should be continued in the context of rigorous biosafety, biosecurity, and risk communication.[157] NSABB reconvened in late March 2012 and recommended the full publication of the University of Wisconsin manuscript and publication of the Erasmus University manuscript after appropriate scientific review and revision, with the caveat that the US government should develop a mechanism to control access to sensitive scientific information.[158] The two manuscripts were published later in 2012.[159,160]

The controversy resulted in an updated US Government Policy for Oversight of Life Sciences DURC, which was released in March 2012.[161] This policy directed federal departments and agencies that conduct or fund life sciences research to do the following:

- review all current or proposed research projects to identify those that could potentially provide knowledge, information, products, or technologies that could be directly misapplied to pose a significant threat to public health and safety, agricultural crops and other plants, animals, the environment, materiel, or national security;
- conduct risk assessments and develop risk mitigation plans addressing experimental design and methods, biosecurity, biosafety, and availability of medical countermeasures;
- review annual progress reports to determine whether DURC results have been generated;
- request voluntary redaction of research publications or communications or classification of research findings; and
- coordinate information regarding DURC projects with the Assistant to the President for Homeland Security and Counterterrorism.

In addition, the Office of Science and Technology Programs is formulating a complementary policy that delineates oversight responsibilities for research institutions receiving federal funds to perform DURC.[162]

In December 2012 NIH hosted a meeting of the influenza research community to discuss guidelines for funding HPAI H5N1 influenza virus gain-of-function research, followed by an opportunity for public comment. The resulting guideline was issued on February 21, 2013,[163,164] and it identified criteria for funding research proposals that may enhance the transmissibility of HPAI H5N1 among mammals:

- the virus anticipated to be generated could be produced through a natural evolutionary process;
- the research addresses a scientific question with high significance for public health;
- there are no feasible alternative methods to address the same scientific question in a manner that poses less risk than the proposed approach;
- biosafety risks to laboratory workers and the public can be sufficiently mitigated and managed;
- biosecurity risks can be sufficiently mitigated and managed;
- the research information is anticipated to be broadly shared to realize its potential benefits to global health; and
- the research will be supported through funding mechanisms that facilitate appropriate oversight of the conduct and communication of the research.

The framework also outlined a review process that includes department-level scrutiny of proposals considered for funding by DHHS agencies.

Five days after the release of the DHHS framework, the ethical, societal, scientific, safety, and security issues raised by DURC were discussed at the international level at WHO. There was consensus that DURC issues are relevant to all nations and multiple stakeholders; management of DURC should take place during all phases of research; ethical considerations are fundamental; and because management of DURC will require a diversity of approaches in different member states, an internationally binding agreement would be difficult, impractical, and not necessarily effective. However, the participants remained open to future international guidelines and suggested that existing international agreements (eg, the BWC, WHO's International Health Regulations [IHR]) could provide a basis for overarching principles. WHO will continue to engage member states and other stakeholders to explore effective approaches.[165]

In the meantime, the influenza research community had already ended its moratorium for scientists using biosafety and biosecurity measures in compliance with its respective national regulations.[166] The subsequent publication of a study completed before

the moratorium using reverse genetics to generate 127 hybrids of HPAI H5N1 and 2009 pandemic H1N1 viruses, of which five were communicable among guinea pigs,[167] again raised questions regarding the medical utility and public health risks of hazardous experiments.[168] In August 2013 proponents of gain-of-function research publicly announced their intention to conduct studies using influenza A H7N9 virus.[169] Concurrently, DHHS gave assurances that research proposals for H7N9 gain-of-function research would undergo rigorous scrutiny by experts in multiple disciplines including biosafety and ethics and final review at the department level,[170] consistent with the February DHHS framework. The a priori publication of H7N9 research goals was seen as a proactive step to enhance transparency and prospective discussion and to prevent a recurrence of the 2011–2012 H5N1 disputes. However, gain-of-function research remains a contentious issue[171] because no certainty exists that laboratory-generated mutants will emerge in nature. The issues generated by potential dual use research will continue to fuel discourse regarding relationships among stakeholders, and optimal policy and technical solutions.[172–179]

Toward Pan-Hazard Preparedness

During the late 1990s the US government launched an ambitious program to enhance biological preparedness at local, state, and federal levels, including measures such as the Presidential Decision Directive-39 (1995), Presidential Decision Directive-62 (1998), and Presidential Decision Directive-63 (1998). The Federal Response Plan (now called the National Response Plan) coordinates federal agencies responding to disasters. The Select Agent List was created to regulate the purchase, shipment, and research of designated microbial agents; lead proponents for the Select Agent list were DHHS and USDA. DHHS was given oversight of health and medical services, and its Office of Emergency Preparedness organized local medical response teams in 125 jurisdictions. Preparations in New York City and other locations included plans and exercises for local incident command; coordinated clinical response; surveillance; and massive distribution of postexposure prophylaxis at multiple distribution centers designed for efficient screening, triage, distribution, and documentation. Federal response teams were organized, staffed, and deployed to large official and public gatherings. CDC established a center for bioterrorism response to enhance state public health laboratories, improve surveillance systems, and improve rapid communication and coordination. The Strategic National Stockpile of key pharmaceutical agents and vaccines was prepared. The Laboratory Response Network, also managed by CDC, provided coordination of testing, sample shipment, and communication between designated local, regional, and reference laboratories. DoD assets integrated into the National Response Plan included USAMRIID for emergency medical consultation and reference laboratory support; the Naval Medical Research Center for laboratory support; the US Marine Corps Chemical and Biological Incident Response Force for reconnaissance, initial triage, and the decontamination of casualties; and the Army Technical Escort Unit for sampling, transport, and disposal of dissemination devices. The Army Medical Department also fielded six regionally based chemical/biological special medical augmentation response teams to deploy within 12 hours to assist local civilian authorities. The National Guard Bureau, under legislative direction from Congress, fielded regional biological response teams initially called rapid agent identification teams, and later renamed civil support teams. Many of these new response mechanisms and agencies were tested in the autumn of 2001.

After the anthrax mailings of 2001, bioterrorism response was strengthened with additional infrastructure and linkages among the emergency response, public health, clinical, and laboratory sectors.[103,104] The Office of Public Health Emergency Preparedness at DHHS was formed to coordinate civilian medical countermeasure development by the National Institute of Allergy and Infectious Diseases, CDC, and DoD, under the leadership of eminent scientists and physicians such as DA Henderson and Philip K Russell.

In April 2004 President George W Bush signed Homeland Security Presidential Decision Directive-10, Biodefense for the 21st Century, which outlined a national strategy for combating biological terrorism and mandated an interagency approach using strengths of various executive branch departments, including the Department of Homeland Security, DHHS, and DoD. Subsequently, the Homeland Security Council and the National Security Council formed an interagency steering committee called the Weapons of Mass Destruction Medical Countermeasures Subcommittee, whose principals were at the assistant secretary level; the group coordinates the various departmental efforts to prevent and respond to weapons of mass destruction attacks. The Department of Homeland Security took the lead on biological threat assessments, and DHHS took the lead on medical countermeasures.

On July 21, 2004, Project Bioshield was initiated as a $6 billion, 10-year program for acquiring new medical countermeasures for the Strategic National Stockpile. This legislation provided a significant funding boost to

the Office of Public Health Emergency Preparedness. Medical countermeasures added to the Strategic National Stockpile include significantly increased doses of botulinum antitoxins; antibiotics to treat anthrax, tularemia, and plague; anthrax adjunctive therapies; and ventilators for respiratory support.

The potential for the malevolent use of genetic engineering to develop novel biological threats with enhance virulence[180] resulted in a shift of technical emphasis from pathogen-specific projects to a global response capability—a threat-agnostic response capacity—to enable responses to outbreaks of any known or genetically engineered biological agents, or novel emerging pathogens. This capability includes flexible technology platforms to enable rapid pathogen identification and characterization, drug target identification, and medical countermeasure development and mass production. An emphasis has been placed on the development of anti-infective therapeutics that has a broad spectrum of activity to enhance their potential utility against a wide range of emerging pathogens. In addition to exploiting highly conserved pathogen targets, proposed approaches have included host-directed anti-infective therapeutics to upregulate innate immunity, antagonize host receptors and processes that are hijacked by pathogens to complete their life cycles, and attenuate sepsis and other pathogenesis pathways.

The National Strategy for Countering Biological Threats[3] proposed an integrated approach to all biological threats, whether from intentional releases (biological warfare or terrorism) or accidental releases (laboratory accidents or unintended consequences of legitimate scientific research) or naturally occurring emerging diseases. The strategy is based on the concept that all of these challenges require a common set of responses (pathogen identification and characterization; patient diagnosis; development, mass production, and distribution of medical countermeasures; medical and public health interventions; risk communication; promotion of ethical standards; professional and legal codes of conduct; and law enforcement). It proposes a pan-sector "all of society" approach that integrates the public at large and the scientific, medical, veterinary, public health, law enforcement, and diplomatic communities. Initiatives have included reorganization of civilian biodefense under the Department of Homeland Security; strengthening of programs under DoD and DHHS (NIH, the Biomedical Advanced Research and Development Authority, CDC) that have multipurpose utility for biological attacks, naturally occurring outbreaks, and other mass casualty disasters; the construction of the Fort Detrick biodefense campus, which includes laboratories for the Department of Homeland Security and NIH as well as a new USAMRIID facility; export controls to regulate exportation of potential dual use technologies; the medical countermeasures initiative to enhance mass production of medical countermeasures; investments to enhance biosurveillance; and federal guidelines for synthetic biology and the use of double-stranded DNA.

The Federal Experts Security Advisory Panel's interagency working group was initiated in 2010 to update 42 CFR Part 73, Possession, Use, and Transfer of Select Agents and Toxins, to prevent intentional or accidental releases of highly virulent pathogens without placing counterproductive regulatory burdens on laboratories that conduct research on CDC select agents. Topics that were considered included revising the list of select agents, physical security measures, laboratory safety, occupational health, and personal reliability. A simplification of the select agent list was proposed, removing or recategorizing agents that are either easy to obtain from their natural reservoirs, or that constitute low risk due to low virulence, low transmissibility, or the availability of medical countermeasures. The Federal Experts Security Advisory Panel developed a comprehensive set of recommendations regarding biosecurity—the presence of physical security measures such as laboratory access controls, closed circuit visual monitoring, etc, and personal reliability—as well as background checks of laboratory workers' law enforcement history, substance abuse, and mental health, with continuing monitoring and periodic reassessments of suitability for continued employment. Robust occupational health programs, with mandatory reporting of illnesses requiring medical intervention, were emphasized to prevent behaviors that could result in accidental or deliberate releases of select agents and to promptly recognize and treat laboratory-acquired infections and prevent their transmission to the general community. The Final Rule (October 5, 2012) included a revised select agent list; physical security standards for laboratories possessing Tier I Select Agents and Toxins; a requirement to conduct pre-access assessments and ongoing monitoring of personnel with access to Tier I agents and toxins; and clarifications of regulatory language concerning security, training, biosafety, and incident response.[118,119]

The optimization of biosafety and biosecurity is an iterative process. USDA's Office of the Inspector General noted that while there had been enhanced compliance with security regulations and inspection processes within the USDA Select Agent program between 2005 and 2012, there had been transfers of *B anthracis* and *Y pestis* samples to unregistered facilities, and access to select agents by a person with an expired security clearance. USDA concurred with recommendations

to clarify restricted access requirements and establish policies and procedures for handling requests for transferring select agents under special circumstances to unregistered facilities.[181] On March 24, 2013, a vial of Guanarito virus (a Tier I Select Agent) was reported missing from the University of Texas Medical Branch at Galveston.[182] On the following day, the Government Accountability Office issued a report concluding that US government interdepartment and interagency biodefense programs using high containment laboratories should improve their coordination. It also recommended that the Office of Science and Technology within the Executive Office of the President conduct periodic assessments of the requirements for, and the number, locations, and missions of high-containment laboratories, and evaluate the need to establish national standards for their design, construction, commissioning, operation, and maintenance.[183]

International efforts include the following:

- outreach by DoD and CDC to enhance surveillance with international partners;
- DoD's Cooperative Biological Engagement Program that builds partnerships to convert former biological weapons programs to peaceful purposes and enhance public health capacity;
- collaborations to strengthen biological defense capacities of partner nations (eg, through the North Atlantic Treaty Organization and the Australia–Canada–United Kingdom–US–New Zealand partnership);
- US government support of BWC confidence-building measures and international public health efforts that may also lead to the early identification and containment of biological attacks (eg, WHO's IHR); and
- WHO efforts to enhance implementation of the IHR and strengthen ties with the World Organization for Animal Health and Interpol.

SUMMARY

The use of microbes and toxins to intentionally cause harm has been attempted repeatedly throughout recorded history. However, military use before the development of modern microbiology was limited, possibly because of the availability of other weapons with more rapid and predictable results.

Following the inception of modern microbiology, several nations began offensive biological warfare programs. Information regarding the history of state-sponsored biological weapons programs is obscured by secrecy, propaganda, and a lack of rigorous microbiologic or epidemiologic data to confirm allegations of use. Disclosures of former national programs underscore the ambitious intent and potential realization of covert state-sponsored programs. However, military deployment has been limited, and never decisive in armed conflict. With the exceptions of alleged German sabotage during World War I, Japanese field trials during World War II, limited deployments by South African and Rhodesian forces, and small-scale covert operations, there are no well-documented biological attacks by nation-states. Deterrents may include poor tactical utility related to multiple variables during production, storage, and delivery; variable incubations and host susceptibilities; availability of medical countermeasures; nuclear deterrence; diplomatic efforts; and political vulnerabilities. The public health disaster at Sverdlovsk, the loss of international goodwill toward the United States following disclosures during the Cold War, and political consequences following the 1996 disclosures by Iraq underscore that the attendant liabilities of state-sponsored biological weapons programs have outweighed potential strategic advantages.

Non-state groups, lone actors, and even members of the medical community have committed bioterrorism and biocrimes. The likelihood of amateurs using homemade equipment to successfully develop and deploy a biological weapon of mass destruction is remote. Terrorists still rely on simple yet effective explosives as their weapon of choice. However, the Aum Shinrikyo program and Al Qaeda aspirations demonstrate intentions to harness modern microbiology for malicious purposes. Although most bioterrorism incidents and biocrimes have had limited results, the 1984 Rajneeshee episode and the 2001 anthrax mailings illustrate that even relatively small-scale attacks can have enormous public health, economic, and social consequences.

Biological weapons have been renounced by 170 states-parties to the BWC for numerous political and strategic considerations. Counterproliferation efforts, including verification of compliance of signatory states, remain challenging. According to an unclassified 2013 US Department of State report, uncertainties exist about activities in Russia, Iran, North Korea, and Syria.[78] These ambiguities, in addition to the miscalculations of the 2002 National Intelligence Estimate, underscore the difficulty of assessing biological weapons programs even through the rigorous efforts of highly dedicated and skilled professionals. These concerns highlight the importance

of strengthening international goodwill and transparency through the BWC and international engagement programs.

The threats of biological weapons have led to new technical strategies:

- a movement from addressing a static list of a limited number of specific pathogens toward a threat-agnostic capability-based approach using flexible enabling technology platforms that can be rapidly adapted to counter novel, unanticipated pathogens;
- broad spectrum therapeutics; and
- versatile response capacities that can be used to counter biological weapons attacks, naturally occurring epidemics, or other mass casualty disasters.

The past decade has also seen efforts to integrate multidisciplinary societal sectors ranging from research to operational response-surveillance; medical care delivery; risk communication; the development, mass production, and stockpiling of medical countermeasures; and planning and exercises at local, regional, national, and international levels. The enhancement of diagnostic platforms, disease surveillance and reporting networks, medical countermeasures, and health delivery systems that can be rapidly adapted as common solution sets to either biological attacks or natural epidemics is essential to cost-effective, economically sustainable disease mitigation in an era of limited resources.

Scientific research on highly virulent pathogens is essential to biodefense and public health—broadly inclusive—to counter biological weapons and novel emerging diseases. Such research inevitably carries risks, including accidental releases, transmission of laboratory-acquired infections to the community, unanticipated consequences of well-intended experiments, and the generation of knowledge that could be misused to execute biological attacks. Even with effective risk management, risk never reaches zero, but can be decreased to an "irreducible minimum" through rigorous biosafety and biosecurity. Steps in the right direction include the formulation and enforcement of standards and regulations for biosafety, biosecurity, and handling of select agents. Risks and benefits should be carefully considered a priori, with engagement of a broad stakeholder community. Risk management must preserve opportunities for scientific creativity and academic freedom and also must be open to unanticipated experimental results that may serendipitously lead to valuable new discoveries, such as the reactogenicity of tuberculin purified protein derivative, that led to the repurposing of a failed therapeutic to a valuable diagnostic reagent, and the fungal contamination of a bacterial culture that led to the discovery of penicillin.

Although technical solutions are essential, they are not sufficient. An understanding of the history of the development and use of biological weapons, as well as analyses of risk perception and misperception, and appropriate or misguided responses to perceived risks requires examination from both technical and sociological points of reference, particularly the sociologies of scientific and policy decision-making. Important issues include the psycho-social milieus that generate biological weapon development and use, and that lead either to effective responses to credible threats or to misinterpretation and over-reaction to legitimate biotechnology.[184]

The late Joshua Lederberg, the 1958 Nobel laureate for medicine or physiology, a pioneer of bacterial genetics and recombinant technology, and an expert opinion leader in the fields of emerging infectious diseases and biological defense,[185] remarked:

> There is no technical solution to the problem of biological weapons. It needs an ethical, human and moral solution if it's going to happen at all. Don't ask me what the odds are for an ethical solution, but there is no other solution.[186]

Value-related paradigms of ethical medical research directed toward the good of humanity, which underlie the preamble of the BWC's appeal "to the conscience of mankind,"[187] and the National Strategy for Countering Biological Threats' emphasis that life sciences research should be used "solely for peaceful and beneficial purposes,"[188] proscribe biological weapons, and may also inform approaches to dilemmas posed by DURC. Proposals to obtain new data, information, and knowledge should be evaluated in the context of wisdom and in its relevance to the advancement of the common good, and be open to the possibilities that human actions may have intrinsic meaning and moral value. History demonstrates that when ethics and science are decoupled, potential outcomes include biological weapons. Ethical considerations are as relevant to basic and applied microbiology as the principle of beneficence is to medical research involving human subjects. Academic freedom must be maximized and ethical constructs must be flexible, yet circumstances exist in which it is appropriate to take principled stands.

Moral principles lead to codes of professional conduct based on a commitment that basic and applied sciences must be value-related—purposely directed toward the benefit of society as their long-term goal

with a caveat to "do no harm."[179] Professional ethics must go deeper than financial disclosures and honest reporting of data to address the value and risks of proposed experiments. Because an experiment can be done—as an achievement outside of a value- and goal-related context—does not mean that it should be done. It is essential to build a culture of responsibility at every level of individual investigators, laboratory institutional review boards, funding organizations, and national authorities considering the permissibility of specific research proposals in the context of purpose, methods, potential unintended consequences, and value to society. Moral principles underlying the BWC and the National Strategy for Countering Biological Threats have found expression in the ethical codes of the American Society for Microbiology and other professional organizations, US government guidelines for synthetic biology and DURC, the Cooperative Biological Engagement Program, support for implementation of the IHR, and NSABB's call for the development and dissemination of ethical codes of conduct.[189]

The use of synthetic biology to produce wild-strain poliovirus illustrates the relevance of ethics to biological weapons proliferation and DURC, and the role of coordinated multidisciplinary approaches for risk mitigation. An intended outcome was to sound an alarm that viruses can be synthetically produced to develop biological weapons; a conclusion was that WHO's goal of polio eradication may be unrealistic and should be reconsidered in view of issues that include the potential reintroduction of synthetic poliovirus as an act of bioterrorism.[136,138] Alternatively, the chemical synthesis of the oral polio vaccine would have demonstrated an innovative cell-free platform for the production of attenuated live viruses for vaccines. This would have been an unambiguously benevolent action and would have supported the investigators' intention to test the hypothesis that live viruses can be synthetically produced. The inductive proposition that synthetic viruses may pose biological weapons proliferation risks would have been obvious. The investigators later directed their platform toward novel approaches to vaccine development[138,190–193]; in the context of altruistic medical research, this could have been their stated objective and technical approach from the outset.

During the timeframe when the synthesis of wild-strain poliovirus was being conducted and reported, WHO was already proposing material and nonmaterial solutions for the contingency of a posteradication outbreak resulting from either bioterrorism or an accidental reintroduction.[194–198] A 2013 WHO strategic plan for the final phase of polio eradication combines multidisciplinary pan-sector approaches including the global incorporation of inactivated polio vaccine into routine immunization programs, coordinated withdrawal of oral polio vaccine, biocontainment of all wild and vaccine strains, enhanced surveillance, a vaccine stockpile for emergency use, communication, and response.[199] The potential abuse of synthetic biology for biological weapons proliferation has not derailed the polio eradication campaign,[199–202] just as the risk of biological warfare using variola did not obviate the goal of smallpox eradication.

Medical capabilities and biomedical research are being linked to diplomacy, commerce, education, ethics, law enforcement, and other activities to enable a common set of multidisciplinary pan-societal sector responses to both biological weapons and the inevitable and dynamic challenges of naturally occurring emerging infectious diseases.[3] Integration of biological defense and public health programs and their mutual development must be continuous to optimize outcomes and maximize efficient utilization of limited resources and because the challenges posed by both biological weapons agents and naturally emerging pathogens are open ended.

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Chapter 2

EPIDEMIOLOGY OF BIOWARFARE AND BIOTERRORISM

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IMPROVING RECOGNITION AND SURVEILLANCE OF BIOTERRORISM

POTENTIAL IMPACT OF ADVANCED MOLECULAR TECHNIQUES ON THE EPIDEMIOLOGY OF BIOWARFARE AND BIOTERRORISM

SUMMARY

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A portion of this chapter has previously been published as: Dembek ZF, Kortepeter MG, Pavlin JA. Discernment between deliberate and natural infectious disease outbreaks. *Epidemiol Infect*. 2007;135:353–371.

INTRODUCTION

Preparing for and responding to biological warfare (BW) or bioterrorism (BT) is a public health issue and falls within the purview of public health professionals, because preparation for natural disease outbreaks has the dual benefit of BW/BT preparation. An understanding of basic epidemiology is needed before, during, and after an event to identify populations at risk, target preventive measures such as vaccinations, recognize an outbreak, track and limit disease spread, and provide postexposure treatment or prophylaxis. Many disease-specific management needs such as vaccination and prophylaxis are discussed elsewhere and are not considered here. Also, agricultural terrorism is discussed in chapter 3. This chapter will focus on detection and epidemiological investigation including distinguishing between natural and intentional events. Brief case studies will be presented to demonstrate important indicators and lessons learned from historical outbreaks. Finally, traditional methods of surveillance and ways to improve surveillance for BW/BT will be discussed.

THE EPIDEMIOLOGY OF EPIDEMICS

Definition

The word epidemic comes from the Greek "epi" and "demos," meaning "upon a mass of people assembled in a public place."[1] An epidemic is defined as the occurrence in a community or region of an unusually large or unexpected number of disease cases for the given place and time.[2] Therefore, a critical foundation is knowing baseline rates of disease to determine whether an epidemic is occurring. This information can be at the local, regional, national, or global level, and can be seasonal. As an example, thousands of influenza cases in January in the United States may not be unusual; however, thousands of cases in the summer may be cause for concern, similar to what was seen with an early summer wave of cases of H1N1 swine variant influenza in 2009. Also, even a single case of a rare disease can be considered an epidemic. With the absence of a woolen mill industry in the United States, any inhalational anthrax case should be highly suspect. Many of the diseases considered as classic BW agents, such as smallpox (considered to be eradicated), viral hemorrhagic fevers, and pneumonic plague are rare, and a single case should be investigated. Determining whether an outbreak occurs depends, therefore, on the disease, the at-risk population, the location, and the time of year.

For an outbreak to occur, three points of the classic epidemiological triangle must be present (Figure 2-1). There must be a pathogen or agent, typically a virus, bacterium, rickettsia, fungus, or toxin, and a host (in this case, a human) who is susceptible to that pathogen or agent. The two need to be brought together in the right environment to allow infection of the host directly by another individual, by a vector, or through another vehicle, such as food, water, or contact with fomites (inanimate objects). The environment must also permit potential transmission to other susceptible hosts. Disruption of any of these three points of the triangle can limit or disrupt the outbreak; therefore, it is important to know and understand the characteristics of the three for any specific disease to control an epidemic. For example, if potential hosts are vaccinated, disease spread would be significantly limited or if the environment is modified, spread may also be limited (eg, cleaning up garbage around a home limits rat food and harborage, and thus minimizes the risk of contact with fleas capable of transmitting plague).[3]

Recognition

Immediate effects on humans and possibly the environment are evident when an explosion occurs or a chemical weapon is released. However, because of the incubation periods of infectious pathogens, release of a BW/BT agent may be silent and the casualties produced after a release may be dispersed in time and space to primary care clinics and hospital emergency departments. Even toxins have latent periods prior to symptom onset. Therefore, the success in managing a biological event hinges directly on whether and when the event is recognized.

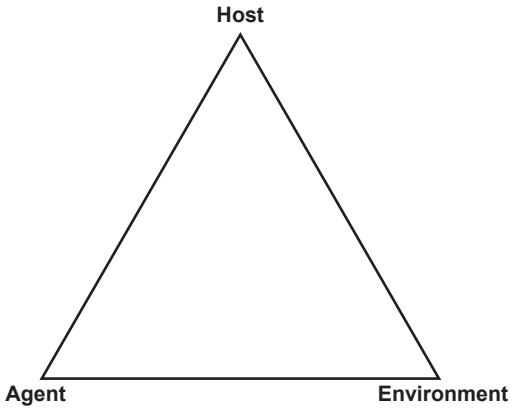


Figure 2-1. The epidemiological triangle

An example of the ramifications of delayed disease outbreak recognition occurred in 1972 in the former Yugoslavia. A single unidentified smallpox case led to 11 secondary cases, also unrecognized. Within a few weeks there was an outbreak of 175 smallpox cases and 35 deaths that led to a massive vaccination effort and border closure.[4] Early disease recognition may have significantly modified the outcome. Modeling studies of a BT-caused smallpox outbreak have shown that the more rapidly a postrelease intervention occurred, including quarantine and vaccination, the greater the chances that intervention would halt the spread of disease.[5–7] When medical professionals identify a new case, it is unlikely that a BW/BT event would be the first cause suspected, especially if the disease presents similar to other diseases that might occur simultaneously, such as influenza. Clinicians generally consider the source to be a common endemic disease at first. Alternative considerations might include a new or emerging disease, or a laboratory accident before considering BW/BT.[8] Therefore, care providers should be familiar with the diseases of BW/BT that could be spread intentionally and maintain a healthy "index of suspicion" to recognize an event early enough to significantly modify the outcome.[9] Furthermore, although the government has generated lists of potential threat agents, public health authorities must be mindful that a perpetrator does not necessarily follow any list and may choose an organism based on access or some other unanticipated reason. Also, a perpetrator might listen to government and other media information, and respond accordingly, thereby undermining a government terrorism response.

Clinicians, hospital infection control personnel, school or healthcare facility nursing staff, laboratory personnel, and other public health workers have a responsibility to notify public health authorities about disease outbreaks. State and local public health officials regularly examine and review disease surveillance information to detect outbreaks in a timely manner and provide information to policymakers on disease prevention programs. Time constraints are inherent in obtaining case report information because of the elapsed time from patient presentation, lab specimen collection and submission, and laboratory testing time, to final disease or organism reporting. Furthermore, the initial BW/BT disease recognition may not come from a traditional reporting partner or surveillance method. Instead, pharmacists and clinical laboratory staff who receive requests or samples from numerous healthcare providers may be the first to note an increase in purchases or prescriptions of certain medications (eg, antibiotics or antinausea or diarrheal agents) or orders for certain laboratory tests (eg, sputum or stool cultures), respectively. Also, because many of the category A high-threat diseases are zoonoses (primarily infect animals), with humans serving as accidental hosts, veterinarians may be the first to recognize the disease in animals prior to the ensuing human disease. Media and law enforcement personnel and other nontraditional reporters of outbreaks may also provide information on a BT event or potential cases. Therefore, it is important for all those different types of individuals to maintain the same index of suspicion as healthcare providers for unusual events in their respective fields.

Potential Epidemiological Clues to an Unnatural Event

It is often not possible to determine the objectives of a BT perpetrator in advance, whether the intent is to kill, incapacitate, or obtain visibility. It also may be difficult to discern how a biological agent was dispersed, whether through the air, in contaminated food or water, or by direct inoculation. In a biological attack, the number of casualties may be small and therefore unrecognized as intentionally infected, especially if the agent is a common cause of disease in the community. In addition, given the agent's incubation period, individuals may seek care from different care providers or travel to different parts of the country before they become ill and seek medical care. Despite the potential for these situations to occur, it is useful for healthcare providers to be aware of potential clues that may be tip-offs or "red flags" of something unusual. Although these clues may occur with natural outbreaks and do not necessarily signal a BW/BT attack, they should at least heighten suspicion that something out of the ordinary is occurring. The following compilation is an illustrative list; however, additional clues may be found elsewhere.[10,11]

Clue 1: A highly unusual event with large numbers of casualties. Although the mention of BW or BT may elicit images of massive casualties, they may not actually occur with a real BW/BT event. Numerous examples of naturally spread illness have caused massive casualties and some BW/BT events have few or no casualties. Nevertheless, the type of large outbreak that should receive particular attention is one in which no plausible natural explanation for the cause of the infection exists.

Clue 2: Higher morbidity or mortality than is expected. If clinicians are seeing illnesses that are causing a higher morbidity or mortality than what is typically seen or reported for a specific disease, this may indicate an unusual event. A perpetrator may have modified an agent to make it more virulent or selected antibiotic resistance in an organism usually

sensitive to antibiotics. Individuals could also be exposed to a higher inoculum than they would normally receive with natural spread of the agent, thus causing higher morbidity or mortality.

Clue 3: Uncommon disease. Many infectious diseases have predictable population and infectivity distributions based on environment, host, and vector factors; yet unnatural spread may occur if a disease outbreak is uncommon for a certain geographical area. Concern should be heightened if the naturally occurring disease requires a vector for spread and the competent vector is missing. For example, if a case of yellow fever, which is endemic to parts of South America and sub-Saharan Africa, occurred in the United States without any known travel, it would be a concern. Natural outbreaks have occurred in new geographical locations including the West Nile virus (WNV) in New York City in 1999.[12] It is important to consider whether the occurrence of these uncommon diseases is natural.

Clue 4: Point source outbreak. For any outbreak, it is useful to develop an epidemic curve demonstrating the timeline of dates when patients developed illness. These curves can have different morphologies depending on whether individuals are exposed at the same time from a single source or over time, and whether the illness spreads from person to person. In an intentional BT event, a point source outbreak curve would most likely be seen[13] when individuals are exposed at a similar point in time. The typical point source outbreak curve has a relatively quick rise in cases, a brief plateau, and then an acute drop, as seen in Figure 2-2. For example, the epidemic curve might be slightly compressed after an aerosol release because infected individuals were exposed more closely in time (ie, within seconds to minutes of each other) compared with individuals becoming ill after eating a common food over a period of hours. Or the inoculum may be greater than what is typically seen with natural spread, thus yielding a shorter incubation than expected. It should also be considered that the spread of a biological agent capable of being transmitted from person to person could result in a propagated (secondary transmission) outbreak, with a case distribution more similar to that depicted in Figure 2-3.

Clue 5: Multiple epidemics. If a perpetrator can obtain and release a single agent, it is also feasible that multiple perpetrators could release single or multiple agents at different locations. If simultaneous epidemics occur at the same or different locations with the same or multiple organisms, an unnatural source must be considered. It must also be considered that a mixture of biological organisms with different disease incubation periods could be released, and thus would cause simultaneous or serial outbreaks of different diseases in the same population.

Clue 6: Lower attack rates in protected individuals. This clue is especially important for military personnel. If certain military units had some type of respiratory protection, such as mission-oriented protective posture gear or high-efficiency particulate air-filtered masks, or stayed in a high-efficiency particulate air-filtered tent and had lower rates of illness than nearby groups that were unprotected, this may indicate that a biological agent has been released via aerosol.

Clue 7: Dead animals. Historically, animals have been used as sentinels of human disease. The storied use of canaries in a coal mine to detect the presence of noxious gases is one example. This phenomenon was observed during the naturally occurring WNV outbreak in New York City in 1999, when many of the local crows, along with the exotic birds at the Bronx Zoo, developed fatal disease.[14,15] Because many biological agents that could be used for BW/BT are zoonoses, a local animal die-off may also indicate a biological agent release that may also infect humans.

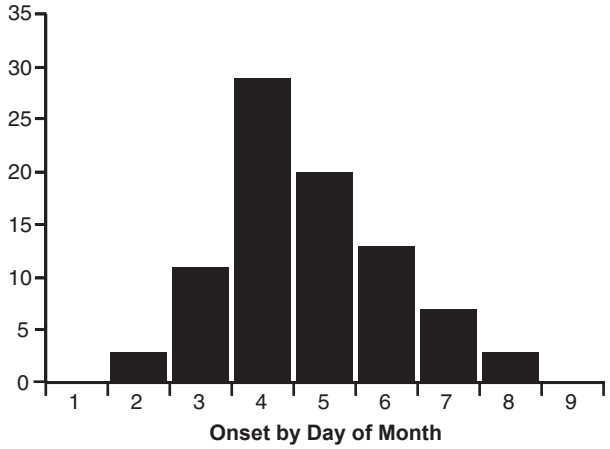


Figure 2-2. Typical point source outbreak epidemic curve

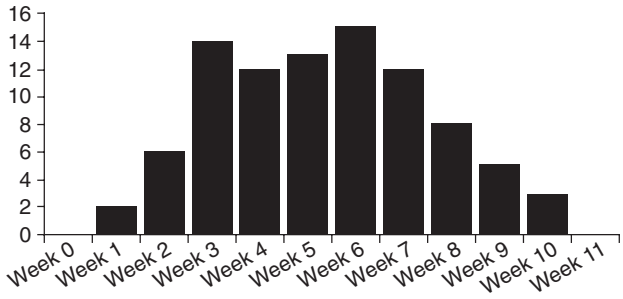


Figure 2-3. Typical continuous common source outbreak epidemic curve

Clue 8: Reverse or simultaneous spread. Zoonotic illnesses exhibit a typical pattern: an epizootic first occurs among a susceptible animal population, followed by cases of human illness. With anthrax, one would expect ill animals to be identified before cutaneous disease in workers processing the animals or before gastrointestinal disease in people who may have eaten meat from the infected animals. After the accidental release of anthrax spores in Sverdlovsk (see description and case review of the 1979 Sverdlovsk anthrax outbreak), an outbreak occurred simultaneously in people and animals downwind of the weapons facility.[16] If human disease precedes animal disease or human and animal disease are simultaneous, then unnatural spread should be considered.

Clue 9: Unusual disease manifestation. More than 95% of worldwide anthrax cases are cutaneous illness. Therefore, a single case of inhalational anthrax should be considered highly suspicious for BW/BT until proven otherwise. The rare exception is an inhalational anthrax case in a woolen mill worker or in someone handling animal skins from endemic areas, which has recently occurred.[17] This logic may be applied to cases of a disease such as plague, where the majority of naturally occurring cases are the bubonic, not the pneumonic form.

Clue 10: Downwind plume pattern. The geographic locations where cases occur can be charted on a geographic grid or map. If the reported cases appear clustered in a downwind pattern, then an aerosol release may have occurred. During the investigation into the anthrax outbreak in Sverdlovsk in 1979 (as examined later in this chapter), mapping out case locations helped to determine that the anthrax cases were caused by an aerosol release rather than a contaminated food source.[16]

Clue 11: Direct evidence. The final clue may be the most obvious and the most useful. Determining the intentional cause of illnesses is easier if a perpetrator leaves a "signature" or direct evidence of a biological attack. Such a signature could be a letter filled with anthrax spores,[18] a spray device or another vehicle for agent spread, or claims by a person or group of a biological attack. It would be useful to compare samples from any found device with the clinical samples obtained from victims to verify that they are the same organism.

EXHIBIT 2-1

GOALSOFANOUTBREAKINVESTIGATION

- Find the source of disease.
- Rapidly identify cases.
- Prevent additional cases through implementation of appropriate control measures.
- Identify strategies to prevent further outbreaks.
- Evaluate existing prevention strategies (including control measures immediately put into place).
- Address public concerns.
- Provide information to leadership to support informed decisions.
- Improve scientific knowledge about the disease.

Outbreak Investigation

It is important to understand the basic goals of an outbreak investigation, as seen in Exhibit 2-1. Any outbreak (a greater than expected number of cases in a specific location, group of people, or time period) should be investigated quickly to find the source of the disease. If an outbreak is ongoing, the source of infection needs to be identified and eliminated quickly. Even if the exposure source has dissipated, all cases should be identified expeditiously, so that ameliorative care can be offered and case interviews can be conducted. Case identification can assist in preventing additional cases, especially with a transmissible infectious disease. Providing information to the public and to leaders is also key to ensure the best public health policies are enacted and followed. With notification of any outbreak, whether natural or intentionally caused, there are standard steps to follow in an outbreak investigation (Exhibit 2-2), although these steps may not always occur in order.[19] The first step is preparation, which involves having the necessary response elements (personnel, equipment, laboratory capabilities) ready and establishing communications in advance with partners who may assist in the investigation. Once an event is ongoing, the second step is to investigate, verify the diagnosis, and decide whether an outbreak exists. Early in an outbreak, its significance and scope are often not known. Therefore, existing surveillance information and heightened targeted surveillance efforts are used to determine whether reported items are cause for concern.

The third step is to define the outbreak and seek a definitive diagnosis based on historical, clinical, epidemiological, and laboratory information. A differential diagnosis can then be established.

The fourth step is to establish a case definition that includes the clinical and laboratory features that the ill individuals have in common. It is preferable to use a

EXHIBIT 2-2

TEN STEPS IN AN OUTBREAK INVESTIGATION

1. Prepare for fieldwork (identify resources).
2. Verify the diagnosis. Determine whether an outbreak exists.
3. Define the outbreak and seek a diagnosis (including specimen collection and testing).
4. Develop a case definition and identify and count cases.
5. Develop exposure data with respect of person, place, and time.
6. Implement control measures and continually evaluate them.
7. Develop the hypothesis.
8. Test and evaluate the hypothesis with analytical studies and refine the hypothesis.
9. Formulate conclusions.
10. Communicate findings.

broad case definition at first and avoid excluding any potential cases too early. Objective clinical features are preferred, such as temperature exceeding 100.4°F, or diarrhea defined as greater than three watery bowel movements per day, as well as laboratory and pathological reports. The case definition enables the investigator to count cases and compare exposures between cases and noncases and compare these with other investigators and regions using the same case definition. To obtain symptom information, it may not be sufficient to look at healthcare facilities only, but also necessary to interview the ill persons and their family members, as well as coworkers, classmates, or others with whom they have social contact. It is important to maintain a roster of potential cases while obtaining this information. Commonly during an investigation, there is a risk of double or even triple counting cases because they may be reported more than once through different means. Key information needed from each ill person, besides identifying information to ensure accurate case counting and ability to contact the cases again if necessary, includes date of illness onset; signs and symptoms; recent travel; ill contacts at work, home, or school; animal exposures; and treatments received. With this information, an epidemic curve can be constructed (see Figure 2-2) that may provide information as to when a release may have occurred, especially if the disease is known, and an expected exposure date based on the typical incubation period, known ill contacts, or geographic risk factors.

Different modes of disease spread may have typical features that comprise an epidemic curve. If there is a common vehicle for disease transmission (such as a food or water source) that remains contaminated, it might be possible to see a longer illness plateau (a continuous common source curve [Figure 2-3]) than is seen with a point source of infection. If the agent is spread person to person, successive waves of illness may be seen as one group of individuals infects a follow-on group, which in turn infects another, and so on (Figure 2-4). With time and additional cases, the successive waves of illness may overlap with each other.

The fifth step is to document potential exposure data. Cases need to be identified and counted. Once cases have been identified, exposures based on person, place, and time can be determined. Obtaining information from individuals who would likely have had similar exposures but are not ill can also help determine the potential cause and method of an agent's spread. Information can be obtained either informally or formally with a case control study. A case control study is a type of study where investigators identify individuals with and without disease and compare their potential exposures or risk factors for disease. With a known exposure, one can also identify exposed and nonexposed populations and determine illness rates with a retrospective cohort study to help determine whether that particular exposure is a risk factor for disease.

The sixth step is to implement control measures as soon as feasible and continuously evaluate them. If necessary, control measures can be quickly implemented and then modified as additional case information becomes available. The seventh step is to develop a hypothesis. Based on the characteristics of the disease, the ill persons, and environmental factors, a hypothesis can usually be generated for how the disease occurred, how it is spreading, and the potential risk to the uninfected. The eighth step is to test and evaluate the hypothesis using analytical studies and refine the hypothesis.

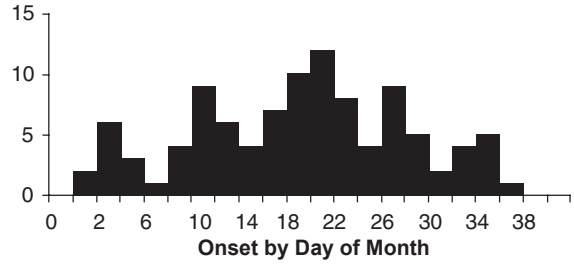


Figure 2-4. Typical propagated (secondary transmission) outbreak epidemic curve

Once developed, it is important to test the hypothesis to ensure it fits with the known facts. Does it explain how all the cases were exposed? It is possible that some outliers may seem as if they should be ill but are not, or some who are ill but have no known exposure. These outliers can sometimes be the key to determining what happened. With preliminary control measures implemented, the hypothesis can be tested formally with analytical studies. Further modifications in control measures might be needed and implemented.

The ninth step is to formulate a conclusion about the nature of the disease and exposure route. Findings can then be communicated (the tenth and final step) through the media or medical literature, depending on the urgency of notification to the public and medical community.

Experience from the anthrax mailings of 2001 indicates that during any BT event, intense pressure will be exerted on public health authorities to provide more information than is available.[20] As stated earlier, these distinct steps may not occur in sequence. It may be necessary to implement control measures with incomplete information, especially if an outbreak is fast moving or has a high morbidity or mortality rate. Whether the control measures appear to limit the disease spread or the casualty toll is the ultimate test of the accuracy of the original hypothesis.

Early in an investigation, it will probably not be known or suspected that an outbreak was unnaturally spread. Therefore, with a few exceptions, the investigation of an unnaturally spread outbreak will not differ significantly from the investigation of a naturally occurring outbreak. Public health authorities will work on both types of outbreaks. The significant difference is that, with a purposeful outbreak, a potential criminal event may have occurred. An additional goal of this type of investigation, under the purview of law enforcement personnel, is to bring the perpetrator to justice. Therefore, law enforcement personnel need to partner with public health officials as early as possible in any suspected BT case.[21]

Public health authorities must become familiar with the use of chain of custody, the process used to maintain and document the chronological history of the evidence, so that medical evidence/clinical samples or environmental samples obtained in the investigation will be admissible in a court of law. Environmental and biological samples can be crucial in determining whether a deliberate release of a pathogen has occurred (see the case study in this chapter about the release of *Bacillus anthracis* in Tokyo by the Aum Shinrikyo).

Although chain of custody is important, public safety should be the primary concern. Public health authorities must also have an open mind for unusual modes of disease spread, being especially careful to ensure their personnel's safety if a potential exposure risk occurs during the investigation. Public health authorities conducting a field investigation should have personal protective equipment and be trained in its proper use, and they should also have access to occupational health resources if pre- or postexposure prophylaxis or monitoring is needed.

EPIDEMIOLOGICAL CASE STUDIES

The following epidemiological case studies are presented to demonstrate the differences between naturally occurring and purposefully created epidemics. Biological attacks and some naturally occurring epidemics of historical significance are considered in the context of BT. Some purposeful BT events have not caused illness; however, some naturally occurring outbreaks were initially considered as potential BT events because of the particular disease or nature of clinical case presentation.

Public health authorities could be held accountable to make a determination quickly as to whether an infectious disease outbreak has been purposefully caused, yet they may lack the necessary information because there may not be clear evidence or responsibility claimed for a BT event. A thorough understanding of how to investigate suspect outbreak occurrences may better enable public health authorities to make difficult public health policy decisions.

Bioterrorism Events

The following section describes BT incidents that occurred in the United States and Japan. None of these events was immediately recognized as having been intentional. The 2001 mail-associated anthrax outbreak and mail-associated ricin attack were recognized within days to weeks. With new sensors installed in mail collection facilities, mailings of ricin in 2013 were recognized immediately. However, for previous BT incidents (anthrax and glanders in 1915, salmonellosis in 1984, and anthrax in 1995), intentionality was not recognized for a year or longer after the initial event.

Anthrax and Glanders—Maryland; New York, New York; and Virginia, 1915–1916

From 1915 through 1918, Germany had a state-sponsored offensive BW program to sabotage suppliers to the Allies directed at draft, cavalry, and military

livestock. Human disease was neither intended nor recorded from these events, although the program could have been expanded to spread zoonotic illness among a target population. Unintended human disease may have occurred but was never recorded. Countries targeted by Germany included the United States, Argentina, Romania, Russia, Norway, and Spain. The German army general staff directed and implemented the biological sabotage program despite official German army doctrine prohibiting such activities. Germany's plans to spread a wheat fungus and contaminate food produced at "meat factories" were dropped.[22] One 1916 German plan never carried out proposed to drop vats of plague cultures from Zeppelins over England.[23]

In April 1915 German-American physician Anton Dilger returned to the United States from Germany with cultures of *Burkholderia mallei* and *Bacillus anthracis*. His intent was to infect horses and mules being shipped from the United States to France and England for use in cavalry and transport. These cultures were propagated and tested for virulence using guinea pigs in the basement of a house (known as "Tony's Lab") rented by Anton and his brother, Carl, in Chevy Chase, Maryland, near Washington, DC.[24] From the summer of 1915 through the fall of 1916, the cultures were used to infect horses and mules in holding pens in docks at the ports of Baltimore, Maryland; Newport News, Virginia; Norfolk, Virginia; and New York, New York. Stevedores working for German steamships were recruited and given 2-inch, cork-stoppered glass vials containing the cultures, in which a hollow steel needle had been placed. These stevedores were instructed to wear rubber gloves while jabbing the animals with the needle. These cultures were also spread to the animals by pouring them into the animal feed and drinking water.[25,26]

Case Review of 1915–1916 Anthrax and Glanders Incidents

Biological Agents: *B anthracis*, gram-positive bacillus; *B mallei*, gram-negative bacillus

Potential Epidemiological Clues: 2, 7, 8

Review: A full assessment of the success of this BW program 90 years later is not possible. German agents claimed that epidemics occurred among the animals shipped from the US ports. However, disease observed among animals might have originated naturally or from stressful holding and shipment conditions.

Few surveillance systems incorporate comprehensive veterinary surveillance. This is an important disease detection vulnerability because many BW agents (ie, *B anthracis*, *Brucella suis*, *B mallei*, *Burkholderia pseudomallei*, *Coxiella burnetii*, *Francisella tularensis*, *Yersinia pestis*, encephalitis, and hemorrhagic fever viruses) can cause zoonotic illness.

Lessons Learned: Veterinarians discovering glanders or anthrax and other US Department of Agriculture (USDA) select agricultural agents in livestock should report these diseases to state health and federal authorities as possible BT indicators.[27,28]

A comprehensive animal surveillance network would include reports from veterinary examinations of farm and companion animals, and from wildlife examinations by state environmental officials and animal rehabilitators. Current animal disease surveillance networks that address these deficiencies include the National Animal Health Laboratory Network[29] and the Centers for Epidemiology and Animal Health,[30] both part of the USDA.

Salmonellosis—The Dalles, Oregon, 1984

A large outbreak of Salmonella cases occurred in and around The Dalles, Oregon, in 1984. This farming community, with a 1984 population of 10,500, is near the Columbia River on the border of Oregon and Washington. Salmonellosis is the second most common bacterial foodborne illness and is underreported by a factor of about 38-fold.[31,32] The average onset period for salmonellosis is about 12 to 36 hours, and the disease manifests as acute gastroenteritis. Fever occurs, anorexia and diarrhea persist for several days, and more severe manifestations may at times occur, especially in very young or elderly persons. Contaminated food (most often poultry) is the principal route of disease transmission.[33]

Given its high incidence in the United States, public health authorities would not normally consider a foodborne salmonellosis outbreak as intentional. It has been estimated that 1.4 million salmonellosis infections occur annually in the United States, resulting in 15,000 hospitalizations and 400 deaths.[34] Therefore, the index of suspicion for an intentional Salmonella outbreak was—and remains—low. However, atypical events associated with this outbreak eventually led officials to realize that this particular disease occurrence was historically different. Two cohorts of cases occurred: (1) from September 9 through 18, 1984, and (2) from September 19 through October 10, 1984. Public health authorities received initial reports of illness on September 17, and local and state health officials interviewed the ill persons. Patronizing two restaurants in the city of The Dalles and eating salad bar food items were commonly cited in these interviews. *Salmonella typhimurium* isolates were then obtained from clinical specimens from the ill persons.[35]

The source for this outbreak was puzzling. Epidemiological analysis revealed multiple items rather than a single suspect item as the cause of the restaurant patrons' illness. This finding is not uncommon either during the initial stages of an investigation of a foodborne disease outbreak (until a suspected food item is identified), or when an infected food handler is identified as the source of the outbreak. Although dozens of food handlers became ill, their time of

symptom onset did not precede those of their customers. As gastroenteritis cases occurred in increasing numbers, health officials imposed a closure of all salad bars in The Dalles on September 25. By the end of the outbreak, 751 salmonellosis cases were identified, with those affected ranging in age from newborns to 87 years, and most were associated with dining in 10 area restaurants. At least 45 persons were hospitalized, but no fatalities occurred.

Bhagwan Shree Rajneesh, a charismatic guru, had established a community for his followers in 1981 at a ranch near The Dalles. These cult members, or "Rajneeshees," attempted to use Oregon's liberal voter registration laws to control zoning and land use restrictions to their advantage. Conflict between the commune and the neighboring traditional community had escalated. To gain political control of the area, the Rajneeshees attempted to influence an election by making voters too ill to vote.[22] Approximately 12 individuals were involved in the plot, and up to 8 individuals distributed *S typhimurium* cultures to the salad bars. After considering the use of several biological agents, including *Salmonella typhi* (the causative agent of typhoid fever) and the human immunodeficiency virus, the Rajneeshees legally obtained cultures of *S typhimurium* (American Type Culture Collection strain 14028) from a commercial supplier and used them to grow bacterial stock cultures. The Rajneeshees first spread Salmonella by contaminating the commune members' hands to greet outsiders, as well as the county courthouse's doorknobs and urinal handles; these efforts did not cause illness. The cult also spread Salmonella cultures on salad bars in area restaurants.

Public health authorities conducted an extensive investigation in response to the salmonellosis outbreak. Authorities identified confirmed cases microbiologically by stool culture of *S typhimurium*, or with the clinical criteria of diarrheal illness and at least three of the following symptoms: fever, chills, headache, nausea, vomiting, abdominal pain, or bloody stools. *S typhimurium* was isolated from 388 patients. In the 4 years before the outbreak, the local health department had collected 16 isolates of Salmonella, 8 of which were *S typhimurium*. No local cases of salmonellosis had been reported in 1984 before August.[35]

The 38 restaurants in The Dalles were grouped according to the number of culture-confirmed customer cases with a single restaurant exposure in the week before symptom onset. Additional ill customers were located through laboratory reporting of clinical specimens or clinician reporting to public health authorities (passive disease surveillance). Press releases were issued to encourage disease reporting by patients and clinicians.[35] Public health officials interviewed ill persons to obtain their symptoms, risk factors, and comprehensive food histories, as well as the names of all persons who had eaten with them at the restaurant. Restaurant employees with the greatest number of cases were interviewed twice and required to submit a stool sample as a condition of continued employment. The state public health laboratory serotyped the Salmonella isolates and performed antibiotic-susceptibility testing on a subset. A representative sample of outbreak isolates was sent to the Centers for Disease Control and Prevention (CDC) for further characterization, during which the outbreak strain was compared with national surveys of human and veterinary isolates. Sanitarians inspected the restaurants, and tap water was collected and analyzed. The local health department and USDA also investigated the food distributors and suppliers used in these restaurants. None was found to have contaminated food, nor was a common supplier found for all of the implicated restaurants.

Many food items served at the salad bars of the restaurants were associated with illness and differed among the restaurants. Illness was associated with eating blue cheese dressing at one of the restaurants. The consumption of potato salad had the greatest association with illness, followed by lettuce. *S typhimurium* was isolated from the blue cheese dressing collected at one restaurant, but not from the dry mix used to prepare the dressing.

The size and nature of the outbreak eventually helped to initiate a criminal investigation. The source and cause of the outbreak only became known when the Federal Bureau of Investigation (FBI) investigated the cult for other criminal violations.[36] An Oregon public health laboratory official accompanying the FBI discovered an open vial containing the original culture strain of *S typhimurium* in the Rajneeshee clinic laboratory in October 1985.[22,35] This strain was indistinguishable from the outbreak strain as isolated from food items and clinical specimens, and records were found documenting its purchase before the outbreak.[35]

Intentional contamination of the salad bars is consistent with the retrospective epidemiology.[35] Eventually two cult members were arrested and served federal prison terms. Despite the Rajneeshees' success of the restaurant-associated BT, the publicity and subsequent legal pressure caused them to abandon subsequent efforts.[22]

Case Review of 1984 Salmonellosis Outbreak

Biological Agents: *S typhimurium*, gram-negative bacillus

Potential Epidemiological Clues: 1, 4, 5, 11

Review: Public health authorities found no statistical association with any single food item.[22] The isolation of *S typhimurium* from the blue cheese dressing, but not from the dry mix used in dressing preparation, should have indicated to authorities the contamination of the prepared dressing that was then served at a salad bar.

The ongoing law enforcement investigation eventually revealed purposeful restaurant food contamination by the Rajneeshees more than a year after the outbreak occurred.

Public health and law enforcement authorities lacked cooperative protocols in 1984; however, law enforcement teams in Oregon worked together with public health.

An outbreak of this magnitude now would initiate a joint inquiry and investigation by public health and law enforcement, increasing chances that the outbreak cause would be identified in a timelier manner.

Lessons Learned: These events illustrate the need to have joint public health and law enforcement investigations and mutual cooperation.

This outbreak shows t yohe importance of the mode of disease spread in discerning the source.

Although not occurring in this case, when different geographic locations are affected, there could be a central supplier of a contaminated product shipped to all the locations. Since there was not a single supplier in this situation, this served as a red flag that multiple contaminations may have occurred.

Anthrax—Tokyo, Japan, 1995

Sarin is a chemical (nerve) agent that causes blocking of the postsynaptic enzyme that degrades acetylcholine, thus leading to excessive salivation, lacrimation, respiratory compromise, and seizures. Many may be familiar with it as a result of its use in the Syrian civil war in 2014. The notorious sarin attacks in a Tokyo suburb, Kameido, in 1994 and 1995, culminated with a sarin release in the Tokyo subway system.[37,38] Less well known is that before its efforts with chemical weapons, the apocalyptic cult Aum Shinrikyo appears to have first invested efforts into producing biological agents and had attempted to use them.[22]

Shoko Asahara, a charismatic guru, built the Aum Shinrikyo cult into a membership of approximately 10,000 individuals with financial assets exceeding $300 million. Aum Shinrikyo's organization mimicked a government entity, with various ministries and departments, including a ministry of science and technology that included graduate-level researchers within modern laboratories interested in developing biological and chemical weapons. *B anthracis* cultures were also obtained and grown into a slurry for use as a biological weapon. This cult may have also investigated the use of *C burnetii* (the rickettsial organism that causes Q fever) and toxic mushrooms. In 1992 a team of 40 cult members, including Asahara, traveled to Zaire to attempt to acquire Ebola virus; the success of these efforts is unknown.

The Aum Shinrikyo experimented with the release of aerosolized biological agents. In June 1993 the cult sprayed *B anthracis* from the roof of one of its buildings in downtown Tokyo. In July 1993 the cult sprayed *B anthracis* from a moving truck onto the Diet (Japan's parliament) and also around the Imperial Palace in Tokyo.

Information about the anthrax releases became public when, during the arraignment of Asahara on May 23, 1996, for the Kameido sarin attack, cult members testified about their efforts to aerosolize a liquid suspension of *B anthracis* to cause an inhalational anthrax epidemic. Their goal was to have an epidemic trigger a world war that would permit Asahara to rule the world.[39] In 1999 a retrospective case-detection survey was conducted to assess the possibility that some anthrax cases may have been unreported. Complaints of odors from neighborhood residents were associated with the anthrax releases. These complaints were retrospectively mapped to provide the geographic areas of the greatest anthrax exposure risk. Physicians at 39 medical facilities serving this area were surveyed. None reported having seen cases of anthrax or relevant syndromes.[39] It is not known whether a similar retrospective examination of anthrax-caused animal deaths was or could have been performed. Danzig and colleagues wrote a comprehensive report that analyzed the Aum Shinrikyo's failures and successes in developing biological and chemical weapons.[40]

Case Review of 1995 Anthrax Releases
Biological Agents: *B anthracis*, gram-positive bacillus
Potential Epidemiological Clues: 11

Review: Technical errors in either the biological agent production or dissemination rendered the attacks harmless. In contrast, there were 12 deaths and about 1,000 hospitalizations from the sarin releases by the Aum Shinrikyo.[37]

Molecular analysis revealed that the *B anthracis* isolates were similar to the Sterne 34F2 strain, the strain of anthrax used in animal vaccines. Dispersal of this type of anthrax (regarded as nonpathogenic for immunocompetent individuals) had little possibility to cause harm to humans.[39]

Even if the strain was pathogenic, the concentration of spores in the liquid suspension is significantly less (104 bacteria/mL) than that considered optimal for a biological weapon (109–1010 bacteria/mL). The viscosity of the suspension was also problematic for successful aerosolization.[39]

The weather on the day of dispersal may have helped prevent infection: spore inactivation resulting from solar radiation could have further reduced the anthrax mix's potency.[39]

Lessons Learned: These experiences show that it is difficult to both create a pathogenic biological weapon and deploy it successfully.

Both health and law enforcement officials should be aware of the possibility for use of more than one biological agent or a combination of agents.

Environmental sample collection and proper storage are important for viability of pathogen cultures.

The then-emerging discipline of forensic molecular biology proved the occurrence of an anthrax release by analysis of archived samples 8 years after the incident.[41] The

contributions of advanced molecular techniques to the detection of BW and BT is examined in the section, Potential Impact of Advanced Molecular Techniques on the Epidemiology of Biowarfare and Bioterrorism, at the end of this chapter.

Shigellosis—Dallas, Texas, 1996

From October 29 through November 1, 1996, 12 clinical laboratory workers at the St Paul Medical Center in Dallas developed severe acute diarrheal illness.[22] *Shigella dysenteriae* type 2 was cultured from the stool of eight of these cases. This strain of shigella is uncommon and, before this outbreak, had last been reported as the source of an outbreak in the United States in 1983. A 13th individual became ill after eating pastries brought home by one of the laboratory workers; this individual also had stool cultures positive for *S dysenteriae* type 2. Five patients were treated in and released from hospital emergency departments and four were hospitalized, but no deaths resulted.[42]

During the subsequent epidemiological investigation,[43] laboratory employees who had worked during the first or third shifts, when the ill employees had worked, were interviewed. The employees stated that an unsigned email sent from a supervisor's computer invited recipients to take pastries available in the laboratory break room. The supervisor was away from the office when the email was sent, and the break room could only be accessed using a numeric security code. The muffins and pastries had been commercially prepared, yet no other cases in the community occurred outside of the hospital laboratory. The ill persons reported eating a pastry between 7:15 AM and 1:30 PM on October 29. Diarrhea onset for the ill laboratory workers occurred between 9:00 PM that day and 4:00 AM on November 1. The mean incubation period until diarrhea onset was 25 hours and was preceded by nausea, abdominal discomfort, and bloating. All who ate a muffin or doughnut became ill (ie, 100% attack rate). No increased risk for illness was found from eating food from the break room refrigerator or drinking any beverage, eating in the hospital cafeteria, or attending social gatherings during the estimated time of exposure to the pathogen.

An examination of the hospital laboratory storage freezer revealed tampering of reference cultures of *S dysenteriae* type 2. The stored reference cultures had each contained 25 porous beads that were impregnated with microorganisms. The *S dysenteriae* type 2 vial contained at that time only 19 beads, and laboratory records indicated that the vial had not been used. *S dysenteriae* type 2 was isolated in virtually pure culture from the muffin specimen, and the same organism was isolated from the stools of eight laboratory worker patients. Pulsed-field gel electrophoresis revealed that the reference culture isolates were indistinguishable from those obtained from a contaminated muffin and the collected stool cultures, but differed from two nonoutbreak *S dysenteriae* type 2 isolates obtained from other Texas counties during that time.

Case Review of 1996 Shigellosis Food Poisonings

Biological Agents: *S dysenteriae* type 2, gram-negative bacillus

Potential Epidemiological Clues: 3, 4, 11

Review: There was a strong epidemiological link among the ill persons, the cultured muffin, and the laboratory's stock culture of *S dysenteriae* type 2.

The pathogen provided important clues because it was known to be uncommon and no research with this microorganism had been conducted at the hospital; therefore, laboratory technicians were not at risk of infection through laboratory error. In addition, no concurrent outbreaks of *S dysenteriae* type 2 were reported nationally at the time.

Pastry contamination during commercial production was unlikely. Shigella contamination by a food service worker during food preparation would have had to occur subsequent to baking because Shigella bacteria would not have survived the heat.

When the epidemiological report was published,[42] it was hypothesized that someone had removed the laboratory culture of *S dysenteriae* type 2 from the freezer, cultured the microorganism and inoculated the pastries, and had access to the supervisor's computer and the locked break room.

On August 28, 1997, a laboratory technician who had access to the laboratory culture stocks and a history of purposeful use of biological agents against a boyfriend, was indicted on three charges of tampering with a food product, and accused of infecting 12 coworkers with *S dysenteriae* type 2. She was subsequently sentenced to 20 years in prison.

Lessons Learned: A match of clinical, food, and laboratory isolates helped to prove an epidemiological link among them. The knowledge that only postproduction tampering of the baked goods could have resulted in their successful contamination assisted with the investigation.

Anthrax—USA, 2001

On October 4, 2001, an inhalational anthrax case was reported in a 63-year-old man in Florida.[44] Public health and government authorities initially misunderstood the nature of inhalational anthrax exposure and assumed that he had contracted the illness by outdoor hunting activities.[45] Two other cases were subsequently identified in Florida, and a fourth case of anthrax—via cutaneous exposure—was identified in a female employee at NBC News in New York City.[43] Investigators then realized that the exposures resulted from anthrax-containing letters placed in the mail. On October 15, Senate Majority Leader Tom Daschle's office received

a letter that threatened an anthrax attack and also contained anthrax spores. The Hart Senate Office Building in Washington, DC, was subsequently closed.[46] By the end of the year, anthrax-laden letters placed in the mail had caused 22 cases of anthrax-related illness (11 inhalational [all confirmed], and 11 cutaneous anthrax [seven confirmed, four suspected]) and five deaths. Almost all anthrax cases were among postal workers and those who had handled mail.[47,48] For two cases, it was difficult to determine exact exposure risk. A 12th cutaneous anthrax case related to these mailings occurred in March 2002 in a Texas laboratory where anthrax samples had been processed.[49,50]

Case Review of 2001 Anthrax Mailings
Biological Agents: *B anthracis*, gram-positive bacillus
Potential Epidemiological Clues: 3, 5, 9, 11

Review: An unprecedented national response occurred involving thousands of investigators from federal, state, and local agencies. Close collaboration was required of all agencies, and the CDC and FBI formed partnerships to conduct public health and criminal investigations.[9]

Public health surveillance to detect previously unreported anthrax cases and determine that no new cases were taking place severely strained public health capacity.[51,52] This outbreak highlighted the importance of containing not only the disease but also public panic.

The Laboratory Response Network, a multilevel network connecting local and state public health laboratories with national public health and military laboratories,[53] served as a lead resource for both identifying and ruling out a potential biological attack.[54] Molecular subtyping of *B anthracis* strains played an important role in the differentiation and identification of *B anthracis*. High-resolution molecular subtyping determined that the anthrax mail-related isolates were indistinguishable and likely came from a single source.[55]

Postal workers and others handling mail were shown to be at risk from the anthrax-containing letters[56] and contaminated postal machinery[57]; therefore, federal and state health officials instituted environmental sampling,[58] cleaning,[59] and protective measures as well as antibiotic prophylaxis.[60] Similar protective actions were taken after discovery of the anthrax spore-laden envelope opened in the Senate Office Building.[45] It was later determined that patients frequently did not complete the recommended prophylaxis duration.[61]

As a direct result of the anthrax mailings, on January 31, 2002, the federal government made $1.1 billion available to the states for BT preparedness.[62] Disease detection and notification efforts, a cornerstone of BT preparedness, have changed dramatically since the incident. Continuing efforts to strengthen the public health workforce should help to better detect, respond, and manage a future BT crisis.[63]

Lessons Learned: An enhanced index of suspicion is necessary for unusual manifestations of BT diseases. Healthcare providers can learn to heighten their index of suspicion and diagnosis early if information is available and they are aware of a disease in a community.

Fine particles of a biological agent can become airborne, thereby contaminating areas and placing persons at risk without direct exposure to the contaminated vehicle. An exposure can occur anywhere along the path of the contaminant, and increased medical surveillance and possibly prophylaxis should be instituted for anyone with potential pathogen exposure.

Risk communication and key messages are important to contain potential public unrest.

Ricin—South Carolina and Washington, DC, 2003–2004

After a terrorist plot to use ricin in England in January 2003,[64] this plant-based toxin (a ribosome-inactivating protein) was found in a South Carolina postal facility in October 2003.[65] Ricin was also discovered in the office of Senator Bill Frist at the Dirksen Senate Office Building in Washington, DC, on February 3, 2004.[66]

On October 15, 2003, an envelope containing a note threatening to poison water supplies with ricin and a sealed container were processed at a mail-processing plant and distribution facility in Greenville, South Carolina. Laboratory testing at the CDC on October 21 confirmed the presence of ricin in the container. State health authorities interviewed all postal workers at the facility, and statewide surveillance for illness consistent with ricin exposure was initiated. The postal facility was closed on October 22, and epidemiological and environmental investigations were conducted. Hospital emergency departments, clinicians, health departments, and the postal facility were asked to report any cases consistent with ricin exposure. State poison control center and intensive care unit charts at seven hospitals near the postal facility were reviewed daily. A medical toxicologist and epidemiologists interviewed all 36 workers at the postal facility to determine whether any were ill, and no postal employees had illness indicating ricin exposure. CDC also conducted environmental testing at the postal facility; all tests were subsequently found negative for ricin.[65]

In 2013 ricin poisoning again became a newsworthy event when ricin-laced letters were sent to President Barack Obama, New York City Mayor Michael Bloomberg, and a gun control lobbyist in Washington, DC. A Texas woman, Shannon Guess Richardson, was arrested and charged in this case, after her confession that she had mailed the letters, and left incriminating evidence that her husband had committed this biocrime.[67]

Case Review of 2003–2004 Ricin Events
Biological Agents: *Ricin communis* toxin
Potential Epidemiological Clues: 3, 11

Review: Ricin is a potent cytotoxin derived from the beans of the castor plant (*R communis*). Ricin will likely continue to be a threat agent because castor beans are grown and used commercially worldwide, and the toxin can be readily extracted.

Ricin is considered to be a more rapidly acting toxin when it is ingested or inhaled than when injected. Treatment for ricin toxicity is supportive care because no antidote exists, and the toxin cannot be removed by dialysis.

Difficulties inherent in responding to a threat of ricin use include the lack of a detection method for locating ricin in clinical samples. A mild ricin poisoning may resemble gastroenteritis or respiratory illness. Ingestion of higher ricin doses leads to severe gastrointestinal symptoms followed by vascular collapse and death; inhalation of a small particle aerosol may produce severe respiratory symptoms followed by acute hypoxic respiratory failure.[68]

Lessons Learned: Any ricin threat should be investigated. As no cases resulted from the above exposures, it is likely that the material used in these incidents was not processed, purified, or dispersed in a manner that would cause human illness.

Biological agents that are readily available in nature remain a threat.

Accidental Release of Biological Agents

The following case studies document the events that transpired after what is understood to be the accidental release of BW agents, *B anthracis*[16] and *Variola major*,[69] in the Soviet Union during the 1970s. The former Soviet Union had a massive state-sponsored biological weapons program, as documented by its former deputy director Ken Alibek in his book, *Biohazard*.[70] This account provides frightening emphasis on the dangers to innocent populations from purposeful biological weapon development.

Anthrax—Sverdlovsk, Soviet Union, 1979

In April and May 1979, the largest documented outbreak of human inhalational anthrax occurred in Sverdlovsk in the Soviet Union (now Ekaterinburg, Russia), with at least 77 cases of disease and 66 deaths. Soviet authorities initially reported the occurrence of a gastrointestinal anthrax outbreak. Gastrointestinal anthrax is an uncharacteristic clinical manifestation from ingesting *B anthracis* spores, although it occasionally occurs in the republics of the former Soviet Union.[16,71] When case history and autopsy results were reexamined by a joint team of Soviet and Western physicians and scientists, it became apparent that the Sverdlovsk outbreak and subsequent deaths had been caused by inhalational anthrax.[16] The geographic distribution of human cases coupled with the location of animal cases indicated that all anthrax disease occurred within a very narrow geographic zone (4 km for the humans, 40 km for the animals) from a point of origin in Sverdlovsk. Historical meteorological data, when combined with this case distribution, demonstrated a point of origin at a military microbiological facility, Compound 19.[16] These data also indicated that the most likely day on which this event occurred was April 2, 1979.[16]

Public health authorities established an emergency commission that directed public health response measures on April 10, 1979, which did not include the Soviet military. A triage response was established at Sverdlovsk city hospital by April 12. Separate areas were designated for screening suspected cases and for treating nonsystemic cutaneous anthrax cases and for intensive care and autopsy. Anthrax illness was not believed to be be transmitted from person-to-person. Those who had died were placed in coffins containing chlorinated lime and buried in a separate part of the city cemetery. Hospital and factory workers were recruited into teams that visited homes of both suspected and confirmed cases throughout the city to conduct medical interviews, dispense tetracycline as a prophylactic antibiotic, disinfect kitchens and patient sickrooms, and collect meat and environmental samples for microbiological testing. Local fire brigades washed trees and building exteriors in the section of the city where most cases were located. Some of the control measures that authorities enacted likely had little value. Stray dogs were shot, and some unpaved streets were paved. Newspaper articles were published, and posters were displayed that warned residents of the anthrax risk from eating uninspected meat or having contact with sick animals. Meat shipments entering the city were examined, and uninspected meat was embargoed and burned. In mid-April a voluntary anthrax vaccination program for healthy individuals aged 18 to 55 years was begun in the part of the city where most of the infected persons lived. Of the 59,000 people eligible to receive anthrax vaccine, about 80% received at least a single dose of the vaccine.[16,72]

Case Review of 1979 Sverdlovsk Anthrax Release
Biological Agents: *B anthracis*, gram-positive bacillus
Potential Epidemiological Clues: 1, 2, 3, 4, 7, 9, 10

Review: In the absence of confirmatory information of an aerosol anthrax release, the public health response was spectacular. Research has estimated that approximately 14% more deaths would have occurred in Sverdlovsk in the absence of the public health intervention that included distribution of antibiotics and vaccination.[72]

The Soviet military's secrecy hid many facts that would have helped physicians to diagnose and treat inhalational anthrax exposure. It is possible that many more individuals

than existing medical records indicate may have become ill and recovered, or died.[73] Ambulance personnel often made an initial case diagnosis of pneumonia.[74]

Government authorities confiscated patient records and autopsy reports from the hospital. Some of these records could have provided invaluable inhalational anthrax medical intervention information from those patients that survived. Along with the absence of an epidemiological investigation at Sverdlovsk, this was a stunning loss of vital information for BW defense purposes.[75]

Former Soviet physicians released important information about anthrax prophylaxis and treatment, some of who took tissue samples and records home at their own considerable personal risk. This information indicated that the incubation period for inhalational anthrax may be as long as 2 months and that an antibiotic course of 5 days likely prolonged the incubation period for illness.[75]

Molecular analysis of tissue samples collected from 11 victims, and retained by Sverdlovsk physicians, indicate that these cases had been exposed to a number of different *B anthracis* strains.[76]

Lessons Learned: Retrospective pathology findings from victims, weather patterns, and geographic mapping can help to determine the outbreak source and also whether it spread.

Public health personnel in Sverdlovsk instituted effective preventive measures before they knew exactly what the exposure was or the cause of the illnesses, and they used information from cases to determine possible exposure routes.

Once the disease agent was determined, prophylactic antibiotics and vaccination and protective environmental measures could be provided.

Studies of Natural Outbreaks for Potential Bioweapon Use

Although the following accounts are examples of naturally occurring outbreaks, some components raise suspicion that they were intentionally caused. Subsequent to the 1999 WNV outbreak in New York City, suggestions were made that Iraqi operatives could have covertly released a biological weapon. These allegations by Richard Preston in the *New Yorker* magazine were based on documentation showing that CDC had provided Iraq with various biological agents from 1984 through 1993, including *Y pestis*, dengue, and WNV,[77,78] together with the fact that the Iraqi government was known to have had a covert biological weapons program.[79] Although never shown to be anything other than an imported disease outbreak occurring in an opportunistic manner, this claim received a lot of political attention. Similar allegations of the covert use of a biological weapon could have been made with other outbreaks, including the 2000 Martha's Vineyard (Massachusetts) tularemia outbreak, and they were made during the 1999 through 2000 Kosovo tularemia outbreak, which occurred during wartime.

West Nile Virus, New York, New York, 1999

An outbreak of an unusual encephalitis was first recognized in New York City in late August 1999. On August 23 an infectious disease physician from a Queens hospital contacted the New York City Department of Hygiene and Mental Health to report two patients with encephalitis. The health department then conducted a citywide investigation that revealed a cluster of six patients with encephalitis in which five had profound muscle weakness and four required respiratory support. CDC's initial clinical tests of these patients' cerebrospinal fluid and serum samples indicated positive results for Saint Louis encephalitis on September 3. More cases of encephalitis in New York City ensued, and because eight of the earliest cases were residents of a 2-square-mile area in Queens, aerial and ground applications of mosquito pesticides began in northern Queens and South Bronx on September 3.[80]

Active encephalitis surveillance began in New York City on August 30 and in nearby Nassau and Westchester counties on September 3. A clinical case was defined as a presumptive diagnosis of viral encephalitis with or without muscle weakness or acute flaccid paralysis, Guillain-Barré syndrome, aseptic meningitis, or presence of the clinical syndrome as identified in earlier cases.[80] Before and during this outbreak, an observed increase in bird deaths (especially crows) was noted in New York City.[14] The USDA National Veterinary Services Laboratory in Ames, Iowa, analyzed tissue specimens taken from dead birds in the Bronx Zoo for common avian pathogens and equine encephalitis. When these test results were negative, the samples were forwarded to CDC, which revealed on September 23 that the virus was similar to WNV in genetic composition.[81] At that time WNV had never been isolated in the western hemisphere.

Concurrently, brain tissue from three New York City encephalitis case deaths tested positive for WNV at the University of California at Irvine. As of September 28, 17 confirmed and 20 probable cases had occurred in New York City and Nassau and Westchester counties, resulting in four deaths. Onset dates were from August 5 through September 16. The median age of the patients was 71 years (range 15–87 years). By October 5 the number of laboratory-positive cases had increased to 50 (27 confirmed and 23 probable). Emergency telephone hotlines were established in New York City on September 3, and 130,000 calls were received by September 28. About 300,000 cans of *N,N*-diethylmetatoluamide (DEET)-based mosquito repellant were distributed citywide through local firehouses, and 750,000 public health leaflets were distributed with information on protection from mosquito bites. Radio, television, and the Internet provided public health messages.[80]

A seroprevalence survey later determined that approximately 100 asymptomatic infections and 30 WNV fever cases occurred for each WNV encephalitis case previously identified in the New York City area.[82]

Case Review of 1999 West Nile Virus Cases
Biological Agents: WNV, a flavivirus
Potential Epidemiological Clues: 1, 2, 3, 7
Review: Although some suggestions were made that this could have been a bioterrorist attack, the appearance of WNV in New York City in 1999 and its subsequent spread to the rest of the United States was most likely a natural occurrence.

Saint Louis encephalitis and WNV are antigenically related, and cross reactions can occur with some serologic testing.[80] Limitations of serologic testing underscore the importance of isolation and identification of virus.[80]

Within its normal geographic area of distribution in Africa, West Asia, and the Middle East, birds do not normally show symptoms when infected with WNV.[83] WNV from this part of the world occasionally causes epidemics in Europe that may be initiated by migrant birds.[84,85] An epizootic that results in the deaths of large numbers of crows may be a clue that either a new population is susceptible to the virus or a new, more virulent strain of a virus has been introduced.[80]

WNV is transmitted primarily by Culex mosquitoes,[86] which contributed to its spread in the United States after the 1999 outbreak.[87]

Genetic testing revealed that the virus was 99% identical to a virus isolated in 1999 from a goose in Israel.[88] Potential routes for WNV introduction include importation of WNV-infected birds, mosquitoes, or ill persons. The New York City area where WNV was prevalent includes two large international airports.[89]

Before this outbreak, death was rarely associated with WNV infection.[90] In patients with WNV encephalitis, computer-assisted tomography often revealed preexisting lesions and chronic changes in brain tissue,[91] perhaps suggestive of the potential for a greater susceptibility to deleterious outcome in elderly persons.

Lessons Learned: This outbreak emphasizes the important relationship among veterinarians, physicians, and public health authorities in disease surveillance, and the importance of considering uncommon pathogens.[90]

The incident is an example of a typical zoonotic disease epidemic pattern—a natural epidemic occurred first among birds, followed by disease in humans.

The origin of outbreaks fitting some of the clues for a biological attack (a new disease for a geographic region) cannot be immediately determined without further investigation. Emerging diseases, whether new for a particular geographic area, like WNV, or a totally new disease (eg, severe acute respiratory syndrome or Middle East Respiratory Syndrome coronavirus), are not uncommon.

Tularemia, Martha's Vineyard, Massachusetts, 2000

During the summer of 2000, an outbreak of primary pneumonic tularemia occurred on Martha's Vineyard, Massachusetts.[92] In July five cases of primary pneumonic tularemia were reported, with onset dates between May 30 and June 22. The Massachusetts Department of Public Health and CDC initiated active surveillance, and 15 confirmed tularemia cases were subsequently identified. A confirmed case was defined as occurring in a visitor or resident to Martha's Vineyard who had symptoms suggesting primary pneumonic tularemia; was ill between May 15 and October 31, 2000; and had test results showing a serum titer of anti-*F tularensis* antibody of at least 1:128 on an agglutination assay. Of these cases, 11 had the pneumonic form of the disease, two had ulceroglandular disease, and two had fever and malaise. Fourteen of the patients were male, and the median age was 43 years (range 13–59). One 43-year-old man died of primary pneumonic tularemia.[92]

Control subjects for a case-control study were obtained by random-digit dialing to Martha's Vineyard residents, enrolling 100 control subjects at least 18 years old that had spent at least 15 days on the island between May 15 and their September interviews. Both ill persons and control subjects were questioned about occupation, landscaping activities, animal and arthropod exposures, recreational and outdoor activities, and general health history and status. Information was obtained about exposure to risk factors between May 15 and the interview, and for 2 weeks before illness for ill persons and 2 weeks before interview for control subjects.[92]

The suspected site of exposure for each patient was visited. Activities that may have led to exposure (eg, lawn mowing and "weed whacking") were reproduced, and environmental and personal air samples were taken. Samples from soil, water, grass, wild mammals, and dogs were also taken. Epidemiological analysis revealed that in the 2 weeks before illness, using a lawn mower or brush cutter was significantly associated with illness. Of all the environmental and animal tissue samples taken, only two were positive for *F tularensis*: (1) a striped skunk and (2) a Norway rat.[92]

Case Review of 2000 Martha's Vineyard Tularemia Outbreak
Biological Agents: *F tularensis*, a gram-negative bacillus
Potential Epidemiological Clues: 1, 2, 3, 9
Review: Caused by a gram-negative bacillus *F tularensis*, tularemia is a rare infection in the United States. Between 2001 and 2010, a median number of 126.5 cases per year (range: 90–154 cases per year) was reported.[91] More than half of all cases reported during these 11 years came from Arkansas, Missouri, South Dakota, and Oklahoma, and most cases were acquired from tick bites or contact with infected rabbits. Higher incidences of the disease have been noted in persons ages 5 to 9 and older than 75, and incidence was greatest among Native Americans and Alaskan natives.[93]

The only other previously reported pneumonic tularemia outbreak in the United States had occurred on Martha's Vineyard during the summer of 1978.[94] During a single week (July 30–August 6) seven persons stayed in a vacation cottage. By August 12, six of these had a fever, headache, and myalgia; and the seventh had a low-grade fever by August 19. A search for additional cases on the island uncovered six other tularemia cases, five of which were pneumonic, and one was ulceroglandular. No source for the disease exposure was discovered, although two rabbits later found dead were culture-positive for *F tularensis*.

Tularemia had been reported sporadically since rabbits had been introduced to Martha's Vineyard in the 1930s,[93] and pneumonic tularemia was first reported in Massachusetts in 1947.[95] Classic research on human tularemia rates showed that very high rabbit populations increase the tularemia hazard.[96]

Hospital clinicians on Martha's Vineyard initially detected this outbreak and recognized tularemia caused pneumonic summer illness,[97] in part based on the experiences with the previous outbreak.[94]

Feldman et al proposed in this outbreak *F tularensis* was shed in animal excreta, persisted in the environment, and infected persons after mechanical aerosolization and inhalation. This is a likely exposure scenario, given the principal form of primary pneumonic tularemia seen in these cases and strong epidemiological association with grass cutting.[92]

A seroprevalence survey conducted in 2001 in Martha's Vineyard demonstrated that landscapers were more likely to have an antibody titer to *F tularensis* than nonlandscapers, revealing an occupational risk for tularemia.[92]

Lessons Learned: Naturally occurring disease can present in the pneumonic form. However, if tularemia were used as a biological weapon, an aerosolized release would probably result in multiple simultaneous cases presenting with the pneumonic form of the disease.[97]

There may also be disease transmission mechanisms (in this example, grass cutting) that are unknown or poorly understood.[98]

Tularemia, Kosovo, 1999–2000

After a decade of political crises and warfare, a large outbreak of tularemia occurred in Kosovo from 1999 through 2000. Tularemia had not been reported in Kosovo since 1974.[99] By April 2000, 250 suspected cases had been identified and spread nationwide, but most cases existed in the western area where ethnic Albanians resided.[100]

Unusual outbreaks of zoonoses or vectorborne disease may readily occur in war-torn or crisis-afflicted regions that have previously been free of these diseases. Historically, outbreaks of typhus, plague, cholera, dysentery, typhoid fever, and smallpox have long been observed in war-torn regions.[101] Among the earliest historic examples is the plague of Athens that arose during the second year of the Peloponnesian War, as described by Thucydides.[102] Speculation may arise that these epidemics were purposefully caused. Many biological agents are zoonotic pathogens,[99] including tularemia, a category A BW pathogen. Purposeful use of this pathogen merits consideration when such an outbreak occurs with a pathogen having the potential to be a biological weapon.[103]

Remarks made by the head epidemiologist at the Kosovo Institute of Public Health about unidentifiable ampoules and white powders discovered near various wells could not be verified and added to a perception of use of a biological weapon by Serbian forces.[99] *F tularensis biovar tularensis* (type A) is highly pathogenic for humans. It is found mostly in North America and has been developed for use as a biological weapon. Disease progression often follows an acute and severe course, with prominent pneumonitis. *F tularensis biovar holarctica* (type B) is less pathogenic and is found throughout the northern hemisphere.[104] To further complicate matters, a 1998 report documented that type A tularemia had been introduced into arthropod populations in the nearby Slovak Republic.[105] The United Nations mission in Kosovo requested that the World Health Organization assist Kosovar health authorities in an epidemiological investigation of the tularemia outbreak. Teams of international and Kosovar public health personnel collaborated in epidemiological, environmental, and microbiological field and laboratory investigations.[106]

Tularemia cases were discovered by both prospective surveillance and retrospective hospital review of a pharyngitis and cervical lymphadenitis syndrome. Ill persons were clinically examined and interviewed, blood samples were taken from suspected cases, and antibiotics were prescribed as appropriate. Rural villagers reported an increase in mice and rats in the summer of 1999. A causal association was suspected between the increased population density of rodents and human tularemia cases. Tularemia is naturally transmitted to humans via small lesions in the skin of persons handling diseased rabbits, ingestion of contaminated water or food, bites of infectious arthropods, or inhalation of infective dusts.[99]

A matched case-control study was conducted with paired households in villages in regions with the greatest number of reported cases. Case households had one or more family members with a laboratory-confirmed case of tularemia as of November 1, 1999. Control households were the two households closest to a suspected case household, having no individuals with the disease, and the person who prepared the family's food was serologically negative for tularemia. Blood specimens were also drawn from all suspected cases. Questionnaires were completed on household

food consumption, water supply, presence of rodents, and condition of wells and food preparation and storage areas. The study period began a month before symptom onset of the first case in the suspected case household. Well water sampling and rodent collection and analysis were performed.

By June 30, 2000, more than 900 suspected tularemia cases had been discovered. From these, 327 were confirmed as serologically positive. The earliest onset of reported symptoms in the confirmed cases was October 1999, with an epidemic peak in January 2000. Confirmed cases were identified in 21 of 29 Kosovo municipalities. Cases were equally distributed by sex, and all age groups were equally affected. Case households were more likely to have nonrodent-proof water sources, and members in these households were less likely to have eaten fresh vegetables. Risk factors for case households included rodent feces in food preparation and storage areas and large numbers of field mice observed outside the house. Of the field samples collected, positive antigen for *F tularensis* was detected in striped field mouse and black rat fecal specimens.

Case Review of 2000 Kosovo Tularemia Outbreak
Biological Agents: *F tularensis*, a gram-negative bacillus
Potential Epidemiological Clues: 1, 3, 5, 9
Review: Clinical and serologic evidence indicate that a tularemia outbreak occurred in Kosovo from October 1999 through May 2000. The case-control study indicated that transmission of tularemia was foodborne based on the associations of illness and large numbers of rodents in the household environment, rodent contamination of food storage and preparation areas, and consumption of certain uncooked foods. Unprotected water that was not boiled likely contributed to the outbreak.

Initial field investigations rapidly demonstrated that a widespread natural event was occurring and likely resulted from the unusual environmental conditions existing in war-torn Kosovo. The principal populations affected by the tularemia outbreak were ethnic Albanians in rural farming villages with limited economic resources. These people had fled during North Atlantic Treaty Organization bombing and Serbian reprisals during the spring of 1999. Refugees discovered bombed and ransacked homes, unprotected food storage areas, unharvested crops, damaged wells, and a rodent population explosion when they returned to their cottages. Both ignorance of infection and lack of hygienic measures contributed to a foodborne infection in the population.[99]

F tularensis can survive for prolonged periods in cold, moist conditions.

A natural decrease in rodent population resulting from the cold winter, food shortages, and the disease itself likely all helped to end the zoonoses.[99]

Although tularemia was not recognized endemically or enzootically in Kosovo before the 1999 through 2000 outbreak, it became well established in a host reservoir. A second outbreak occurred there in 2003, causing more than 300 cases of oropharyngeal tularemia.[107]

Historically, war in Europe caused tularemia outbreaks. During World War II, an outbreak of more than 100,000 cases of tularemia occurred in the Soviet Union,[108] and outbreaks with hundreds of cases following the war occurred in Austria and France.[107]

Lessons Learned: War provides a fertile ground for the reemergence of diseases and potential cover for BW agent use that is plausible and may go unrecognized as a BW event. An extensive epidemiological investigation must be conducted to conclude or disprove that a BW event has occurred.

Q Fever, Iraq 2005

Q fever is a zoonotic disease caused by *C burnetii*, a bacteria found worldwide. Human cases occur from inhalation of aerosols or windborne dust contaminated with *C burnetii* from birth products, milk, urine, and feces of infected animals—most frequently cattle, camel, goats, and sheep. Infections can also occur from ingesting raw milk or eggs as well as tick bites or human-to-human transmission.[109] Due to the bacteria's ability to survive in harsh environmental climates and its high infectivity, there is concern of its use as a biological weapon. The United States developed Q fever as a biological weapon before ratifying the Biological Weapons Convention. The CDC classifies *C burnetii* as a Category B agent.

From June 18 to July 10, 2005, 22 of 38 Marines (58%) from a single platoon in Al Asad, Iraq, experienced a febrile illness.[110] All patients had a rapid onset of fever and chills, and the majority had headache, respiratory, and gastrointestinal symptoms. The patients were diagnosed with upper respiratory infection or atypical pneumonia because there was no diagnostic capability. Subsequent testing was negative for multiple respiratory pathogens. Follow-up serologic testing 6 weeks later on 9 of the affected patients revealed positive Q fever immunoglobulin for all 9, with 10 unaffected persons from the same unit negative for antibody.[110]

After confirmation of Q fever, the researchers distributed follow-up questionnaires to the company that included the affected platoon. They found an association between infection and exposure to ticks and a trend toward association with exposure to camels and the birth of both sheep and dogs. Although the authors did not have a sufficient sample size to confirm all risk factors, they hypothesize that this particular platoon may have sought shelter in an area that was heavily infected secondary to recent animal inhabitation and birthing or ticks.[110]

Before this outbreak, Q fever cases had been reported in US service members deployed to Iraq. An evaluation of 62 cases of pneumonia in 2003 found eight had seroconverted with Q fever antibody,[111] and an additional four diagnosed cases in 2003 and 2004 were reported.[112,113] Three cases of Q fever occurred in US forces in Iraq during the first Persian Gulf War (1990–1991).[114] Since the 2005 outbreak in the Marines, more cases have been reported, and two serosurveys have been performed. One serosurvey revealed 10% of 909 military personnel hospitalized during deployment in 2003–2004 with symptoms compatible with Q fever seroconverted,[115] and another serosurvey studying the same company affected in the outbreak in 2005 found seroconversion in 7.2% of 279 tested.[116] The British military has also published occurrences of Q fever in deployed forces, including 26% of "Helmand Fever" cases caused by Q fever in Afghanistan.[117]

Surveillance of deployed military working dogs in Iraq revealed no seroconversions in 2007–2008, compared to a 5.5% seroconversion in feral dogs.[118] This lack of infection is probably secondary to tick control and doxycycline prophylaxis for the military working dogs.

Case Review of 2005 Q fever cases

Biological Agent: *C burnetii*, gram-negative, facultative, intracellular coccobacillus

Potential Epidemiological Clues: 1, 4

Review: An attack rate of 58% occurred in one platoon. Although the research team was unable to determine exact movements of the platoon, it is likely they had an exposure different from the other platoons.

A relatively short epidemic curve, especially with a long and variable incubation period for the pathogen, suggests a point source. This outbreak probably resulted from an isolated exposure over a short time period.

It is a disease of relatively high severity, had an unknown cause at time of outbreak, and can raise concern about potential intentional cause.

Q fever is considered a potential bioweapon and a cause for concern.

Lessons Learned: All medical personnel should know what diseases are endemic in the area and previous history in deployed forces.

Cases should be reported immediately to allow dissemination of recommended diagnostics and treatment. In this case, the Armed Forces Infectious Disease Society published a set of practice guidelines for diagnosis and management of Q fever to assist deployed medical personnel.[119]

Investigate outbreaks of disease, even after resolution. Knowledge obtained will assist in preventing, recognizing, and rapidly treating future cases.

EPIDEMIOLOGICAL ASSESSMENT TOOL

It is useful for public health authorities to determine whether an infectious disease outbreak is intentional. Grunow and Finke developed an epidemiological assessment tool to rule out biological agent use during infectious disease outbreaks.[98] This assessment tool's relevance was demonstrated by analysis of the 1999–2000 Kosovo tularemia outbreak.[99] In their evaluation scheme, each assessment criterion can be given a varying number of points dependent on its presence and characteristics. There are two types of evaluation criteria: (1) nonconclusive and (2) conclusive. The most significant nonconclusive criteria include a biological threat or risk, special aspects of a biological agent, a high concentration of biological agent in the environment, and epidemic characteristics. Conclusive criteria include the unquestionable identification of the cause of illness as a BW agent (eg, demonstrating modifications that make the agent different from its naturally occurring equivalent, such as stabilizers or physical modifications) or proof of the release of such an agent as a biological weapon. With conclusive criteria, additional confirmatory information is unnecessary.[99]

According to Grunow and Finke's nonconclusive criteria, a biological risk may be considered if a political or terrorist environment exists from which a biological attack could originate:

- **Biorisk**. Are BW agents available, with the means for distribution, and the will to use them? Or can an outbreak be explained by natural biological hazards, or the changes incurred by military conflict?
- **Biothreat**. Does a biological threat exist by virtue of a group having a BW agent and credibly threatening to use it?
- **Special aspects**. Is there plausible evidence of purposeful manipulation of a pathogen?
- **Geographic distribution**. Is the disease's geographic distribution likely given its locale? With the advent of a nonendemic pathogen, a thorough evaluation should include epidemiological, epizootic, ecological, microbiological, and forensic analysis.
- **Environmental concentration**. Is there a high environmental concentration of the pathogen?
- **Epidemic intensity**. Is the course of illness relative to disease intensity and spread in the population expected in naturally occurring illness?
- **Transmission mode**. Was the path of disease transmission considered naturally occurring? The appearance of a naturally occurring epidemic in itself does not rule out the purposeful use of a BW agent.

- **Time**. Was the seasonal timing of the epidemic unusual?
- **Unusually rapid spread**. Was the spread of the epidemic unusually rapid?
- **Population limitation**. Was the epidemic limited to a specific (target) population? If certain persons were given prior warning of a BW attack, then they may protect themselves, as compared to naïve target populations.
- **Clinical**. Were the clinical manifestations of the disease to be expected?

The Grunow-Finke epidemiological assessment procedure (Table 2-1) was used to evaluate the case studies presented in this chapter. To use the assessment tool uniformly for all the events described in this chapter, some artificial constraints were placed on the analysis. For this exercise, only nonconclusive criteria were used because the use of conclusive criteria may have excluded many of the case studies with a retrospective assessment. During an outbreak investigation, however, epidemiological investigators would also initially use the nonconclusive evaluation criteria. With

TABLE 2-1

EPIDEMIOLOGICAL ASSESSMENT AND EVALUATION OF CASE STUDY OUTBREAKS

Nonconclusive Criteria	Assessment (possible points)	Weighting Factor	Maximum No. of Points	1915 Anthrax Eastern USA	1971 Smallpox Aralsk	1979 Anthrax Sverdlovsk	1984 Salmonella Oregon	1995 Anthrax Tokyo	1996 Shigella Texas
Biorisk	0–3	2	6	4	4	4	6	6	0
Biothreat	0–3	3	9	0	0	0	0	6	0
Special aspects	0–3	3	9	6	6	6	3	0	6
Geographic distribution	0–3	1	3	3	3	3	2	3	2
Environmental concentration	0–3	2	6	6	0	6	0	6	0
Epidemic intensity	0–3	1	3	3	3	3	3	0	3
Transmission mode	0–3	2	6	6	2	6	4	0	0
Time	0–3	1	3	3	3	3	1	0	1
Unusually rapid spread	0–3	1	3	3	1	3	3	0	3
Population limitation	0–3	1	3	1	0	1	0	0	3
Clinical	0–3	1	3	3	3	3	0	0	1
Score			**54**	**38**	**25**	**38**	**22**	**21**	**19**

Nonconclusive Criteria	1999 WNV NYC	1999 Tularemia Kosovo	2000 Tularemia Martha's Vineyard	2001 Anthrax USA	2003 Ricin USA	2005 Q Fever
Biorisk	6	2	0	6	6	2
Biothreat	6	3	0	6	9	6
Special aspects	0	0	0	9	0	0
Geographic distribution	3	3	3	3	3	0
Environmental concentration	4	4	4	6	6	0
Epidemic intensity	3	3	3	3	0	1
Transmission mode	2	2	6	6	0	0
Time	1	0	3	3	0	0
Unusually rapid spread	3	1	3	3	0	1
Population limitation	0	0	2	3	0	3
Clinical	1	1	3	3	0	0
Score	**29**	**19**	**27**	**51**	**24**	**13**

NYC: New York City
USA: United States of America
WNV: West Nile Virus

the exception of the 2001 anthrax and 2003 ricin events, none of the outbreaks described had been positively identified as having been caused by a biological agent until sometime after the events had occurred.

Grunow and Finke provide the following cut-off scores for nonconclusive criteria with respect to the likelihood of biological weapon use:

- unlikely (0%–33% confidence): 0 to 17 points;
- doubtful (18%–35% confidence): 18 to 35 points;
- likely (67%–94% confidence): 36 to 50 points; and
- highly likely (95%–100% confidence): 51 to 54 points.

Based on this scoring, only the 2001 anthrax mailings would be considered as highly likely to have been caused by a BW agent. The 1915 and 1979 anthrax events qualify as likely to have been caused by a BW agent. All of the other case study scenarios are either doubtful or unlikely to have been caused by a BW agent.

The authors conducted this evaluative exercise by consensus of opinion. Although subjective, the exercise underscores the challenges facing epidemiologists to determine whether a BT/BW event has occurred, unless direct evidence indicates a purposeful event, or someone credibly claims responsibility. The basic epidemiological principles described earlier in this chapter (including those needed for disease recognition) to determine the occurrence of an unnatural event, and for basic outbreak investigation, are the foundation of infectious disease response and control. Public health authorities must remain vigilant to quickly and appropriately respond to any infectious disease event.

IMPROVING RECOGNITION AND SURVEILLANCE OF BIOTERRORISM

Existing disease surveillance systems may not be sensitive enough to detect a few cases of illness, unless they are legally reportable diseases that have confirmed laboratory diagnoses. However, even before confirmed diagnoses, disease reporting can be initiated upon patient presentation to healthcare providers with initial diagnoses, laboratory testing, and the reason provided by the patient for the hospital visit. Clinicians, laboratories, hospitals, ancillary healthcare professionals, veterinarians, medical examiners, morticians, and others may be partners in reporting diseases to public health authorities.

If a medical surveillance system first detects a biological attack, there may already be a significant number of cases, and the available time to prevent further illness is short or perhaps already over. The point of release is the earliest detection point of a biological event. Some disease exposures could be prevented through publicized avoidance of the area at risk, prophylactic medication use, or vaccination of those exposed, coupled with immediate disease recognition and patient treatment. The Department of Homeland Security's BioWatch program has deployed biological detectors in major urban centers nationwide to detect trace amounts of airborne biological materials[120,121] to help determine the presence and geographic extent of a biological release to focus emergency public health response and consequence management. Such detectors could be of great utility when pre-positioned at large well-publicized gatherings or in cities that may be the greatest targets for terrorist activity.

Although deployed sensors may detect an agent's release, the infinite number of venues coupled with limited resources to position sensors and analyze air samples minimizes the chances that an agent release will be detected. In most instances, the earliest opportunity to detect an attack will be by recognizing ill patients. Depending on the agent, the mode of dissemination, and the number exposed, initial cases will present in different ways. If the disease is severe, such as is possible with category A biological agents, one properly diagnosed case will launch an investigation, as seen during the 2001 anthrax attacks.[47]

Even if the cause is initially unknown, extremely severe or rapidly fatal cases of illness in previously healthy individuals should be reported to public health authorities. If many people are exposed, as would be expected with a large aerosol release of a biological agent, an overwhelming number of people may eventually visit hospital emergency departments and outpatient clinics. Even with less severe disease, such cases should be recognized and quickly reported.

However, in the absence of confirmed laboratory diagnoses or high attack rates, infectious disease outbreaks are often not reported. If the disease is not rapidly fatal or cases are distributed among a variety of healthcare practitioners, it may not be readily apparent that a disease outbreak is under way. Therefore, there is a need for better awareness of the health of communities—a way to quickly detect shifts in potentially infectious diseases, whether of bioterrorist origin or not. This need has been recognized and has resulted in the proliferation of what are commonly known as syndromic surveillance systems.

Syndromic surveillance has been defined as the ongoing, systematic collection, analysis, and interpretation of data that precede diagnosis and can indicate a potential disease outbreak earlier than when public

health authorities would usually be notified.[122] The data used in syndromic surveillance systems are usually nonspecific potential signs and symptoms of an illness spectrum indicating that disease may be higher than expected in a community. These data can be from new or existing sources.[123] For syndrome surveillance of BT, the emphasis is on timeliness, with automated analysis and visualization tools such as Web-based graphs and maps. These tools provide information that initiates a public health investigation as soon as possible.[124]

Numerous regional and national syndromic surveillance systems have been developed, including programs that rely on data collected specifically for the surveillance system and those that use existing medical data (eg, diagnostic codes, chief complaints, nurse advice calls, ambulance runs) and other information (eg, pharmacy sales, absenteeism, calls to poison control centers, Internet searches for specific symptoms or pathogens, participatory epidemiology where people voluntarily provide information to a system like Flu Near You[125] or even scanning Twitter feeds and other social media sites for the use of terms related to illness) to detect changes in population health. Systems that use active data collection can be "drop-in" (those instituted for a specific high-threat time) such as those performed immediately after September 11, 2001,[126–128] or during large gatherings for sports (eg, the Olympics) or other events,[129] or they can be sustained systems for continuous surveillance.[69,130] Systems that require new data entry benefit from greater specificity in the type of syndromes and illnesses reported, but they require extra work and are difficult to maintain. Systems that use existing data can be less specific, especially with information taken from behaviors early in the disease, such as over-the-counter pharmacy sales, absenteeism, Internet searches, and social media use. However, these programs have the large advantage of continuous data streams that are not dependent on provider input or influenced by news reports of disease rates. Such systems (examples of which are described below) have become standard in many health departments, the military, and CDC.

In the US Department of Defense, the Electronic Surveillance System for the Early Notification of Community-based Epidemics uses outpatient diagnostic *International Classification of Diseases, Ninth Revision* codes, chief complaints, radiology and laboratory tests, and pharmacy prescriptions to track disease groups in military beneficiaries. Temporal and spatial data are presented through a Web-based interface, and statistical algorithms are run to detect any aberrations that could indicate a disease outbreak.[131] This system is available for all permanent US military treatment facilities worldwide. Some local and state health departments use civilian versions of the Electronic Surveillance System for the Early Notification of Community-based Epidemics. Other civilian systems, such as the North Carolina Disease Event Tracking and Epidemiologic Collection Tool[132] and various software packages made available by the Real-time Outbreak and Disease Surveillance Laboratory at the Department of Biomedical Informatics at the University of Pittsburgh,[133] and the EpiCenter application[134] also use syndromic information from emergency departments, 911 calls, ambulance runs, and poison control center calls to monitor the health of populations.

CDC has developed the BioSense 2.0 program using national data sources such as the US Department of Defense and Department of Veterans Affairs outpatient diagnostic codes, state and local emergency department visits, and laboratory test orders from commercial vendors to track disease patterns nationwide. The information is provided in a Web-based format to health departments.[135] Algorithms are run on the data and send out an alert when levels of medical visits or laboratory test orders exceed those expected. The information is presented in temporal and spatial format, allowing the health department to track disease based on the patient's home zip code. The BioSense 2.0 goal is to facilitate sharing of automated detection and visualization algorithms and promote national standards.

Despite the proliferation of systems, there are definite limitations in the ability to detect bioterrorist attacks using syndromic surveillance. Some have argued that even if syndromic surveillance could detect an outbreak faster than traditional methods, the advanced warning may not assist with disease mitigation.[73] The warning may not be early enough or effective countermeasures may not be available. In addition, although nonspecific data such as absenteeism and social media may provide some early warning, it is very difficult to institute preventive measures without more specific information. However, nonspecific data can still serve as an early indicator, prompting authorities to monitor specific data sources more carefully.

Most importantly, because a BT attack can present in a variety of ways depending on the agent, population, method of dispersal, and environment, it is impossible to predict how any individual surveillance system will perform. It is generally agreed that most syndromic surveillance systems will not detect a few cases of disease, but they can assist in detecting more widespread disease increases and assessing the population impact, an outbreak's spread, and the success of mitigation efforts. The coverage area of the surveillance system is crucial in determining outbreak detection sensitivity in any part of a community. In the future, syndromic surveillance will probably be based on national models

such as BioSense 2.0 and use readily available electronic databases. Local health departments could then build on a national system using local data that can improve population coverage. Future disease monitoring and reporting systems need to be seamlessly integrated with other traditional disease surveillance systems. Ideally, these systems should also help to educate clinicians on the importance of maintaining a high index of suspicion and to promptly report unusual diseases or disease clusters to public health authorities.

POTENTIAL IMPACT OF ADVANCED MOLECULAR TECHNIQUES ON THE EPIDEMIOLOGY OF BIOWARFARE AND BIOTERRORISM

In addition to the use and application of syndromic surveillance for the detection of shifts in potentially infectious diseases, advances in technologies used for both disease diagnosis and surveillance are helping scientists and healthcare and public health professionals more quickly determine what is causing or has the potential to cause illness.[136–139] These technological advances, which include multiplex polymerase chain reaction, immunoassays, arrays, and even next-generation sequencing, allow a more accurate determination of not only the pathogen,[138,139–142] but also the presence of mutations or other factors that distinguish the organism(s) from previous outbreaks or near neighbors[143] and have the potential to result in more severe disease. These techniques have identified several emerging infectious diseases.[144–146]

Many of the technologies listed have been available for 30 years or more[147,148]; however, the increased speed and multiplex capability, lower cost, and greater application of the technologies as surveillance tools, combined with enhanced surveillance reporting systems, create a more likely environment for the detection of a possible natural or intentional biological event.[149–150] Specifically, the more routine use of sequencing has significantly affected biological sciences and has the potential to be influential in the arena of the epidemiology of biowarfare. Ten years ago the cost and sample-to-result time of sequencing were prohibitive for routine use. However, the cost and processing time continues to decrease, making the accessibility to sequencing more universal and easily adaptable for inclusion in pathogen identification and characterization.[151] In 1990 the National Institutes of Health and Department of Energy initiated the human genome project, which required 10 years to publish a working draft and cost millions of dollars.[152,153] A viral or bacterial genome can be sequenced in a few hours and can cost as little as $100 per isolate.[151,153–157] The use of sequence technology has been instrumental in not only pathogen detection and characterization, including mutations that increase morbidity and mortality, but also in the development of detection and diagnostic assays and therapeutic and prophylactic solutions and/or countermeasures.[155]

Most recently, sequencing was used in the Middle East Respiratory Syndrome coronavirus outbreak to identify the source of the disease, determine the distinction from severe acute respiratory syndrome corona virus,[143] and develop polymerase chain reaction detection and diagnostic capabilities.[23] Sequencing was also used in the H7N9 and H1N1 influenza outbreaks,[158,159] and in the *Escherichia coli* O104:H4 in Germany in 2011[160–162] to assist with identifying the causative agent and developing possible countermeasures. Although it appears as though these events have all been naturally occurring, the addition of characterization information in the form of sequence has allowed researchers to go back and look for possible index cases and the source or reservoir for the outbreak in humans. Rapid sequencing may also facilitate a more rapid vaccine development, as demonstrated in the use of novel techniques for influenza vaccine production.[163] The use of sequencing will continue to assist scientists and public health professionals in their search for not only the reservoir, point of exposure, possible nefarious intention, and comparison with currently known and well characterized diseases, but will also assist in limiting the spread of the disease and possible prevention of future outbreaks by identifying potential zoonotic crossover before it even occurs.[164–167]

Many organizations are conducting surveillance globally with the goal of predicting and preventing the next outbreak or pandemic, often in zoonotic sources.[164,165] The US Agency for International Development,[168] the US Department of Defense, and both for-profit and nonprofit, nongovernmental organizations are all engaged in surveillance efforts using some of these technological advancements to identify the next potential source of an outbreak and develop detection and prophylactic or therapeutic solutions and other nonmedical countermeasures to prevent such an event, or at the very least, to be well prepared to respond robustly and quickly.

However, not all uses of advanced technologies have been without controversy. One recent example of the use of sequencing in the creation of a potential BW agent came in late 2011 and continues today.[169–171] Flu researchers Ron Fouchier, of the Erasmus Medical

Center in The Netherlands, and Yoshihiro Kawaoka, of the University of Wisconsin-Madison, engineered more transmissible strains of H5N1, and, more recently, have focused on H7N9.[172,173] They believe genetic engineering can be used to determine which—if any—mutations accelerate the spread of influenza between mammals.[173–175] Additionally, scientists claim genetically modifying the H7N9 virus in the lab will help drive efforts to develop pandemic drugs and vaccines, and result in better preparedness and response.[175] However, not all scientists agree with the type of research being conducted, including infectious disease specialist Adel A F Mahmoud, of Princeton University.[171] Some scientists worry that these strains could escape the laboratory and possibly kill millions, or get in the hands of the wrong people.[173] Even the US National Science Advisory Board for Biosecurity became involved in this debate and has issued several rulings and restrictions on publication of information from this type of research. Dual use research considerations are also being carefully evaluated in some of these instances to ensure that the global populace is protected from potential harm.

There are other limitations with this type of information gathering and sharing. As seen during the *E coli* outbreak in Germany, when an initial error is made in the suspected source of the outbreak (in this case erroneously stated to be from Spanish cucumbers)[176] the information can seriously and detrimentally affect a nation, manufacturing or processing group, or product identified as the source.[177] Although the initial source of the outbreak was suspected based on epidemiological investigation and early molecular testing, the desire to release the information superseded molecular validation of the suspected outbreak source information[178]; it was not until the results obtained using advanced molecular techniques[160,179,180] combined with further epidemiological investigation identified the more likely outbreak source.[175,181] Additionally, some nations may not approve the release of information regarding an outbreak or may not allow other scientists to continue surveillance or investigations into the source if they feel their economy or other factors such as national security may be threatened. The existence, or lack thereof, of surveillance efforts, systems, and software solutions may also hinder the transfer of information regarding a potential outbreak or emerging infectious disease.[150]

The use of high throughput screening and sequencing technologies can also be instrumental in detection of anomalies indicative of not only natural mutation and resistance, but also engineered and intentional activities.[180,181] The addition of virulence factors such as plasmids that are not typical to given organisms, but convey greater morbidity, communicability, and so forth can be a potential sign of human manipulation. Phylogenetic comparison with known pathogens can not only narrow prevention and treatment options, but also can highlight a possible unnatural combination of strains. Sequence information can even be used to generate a pathogen of interest de-novo, without the pathogenic element, allowing for possible manipulation of once pathogenic organisms in a lower class safety environment and additional options for assay and countermeasure development.[182–184] However, this capability also allows for generation of dangerous pathogens with the proper authorization.[185] Although the knowledge obtained from sequencing can be very beneficial, it has the potential to cause harm if it falls into the wrong hands or is not accurate and does not get reported to the appropriate public health professionals.

However, as evidenced in the last few years when several anthrax and plague cases were detected in patients in the United States, advanced technologies can rapidly assist an epidemiologic investigation. Public health and laboratory officials moved quickly to investigate and determine the source of these infections; and using a combination of molecular techniques and epidemiological outbreak investigation, they found none were suspected to be intentionally caused.[186–193] The addition of advanced molecular techniques can lead to faster diagnosis, treatment, and determination of intent or origin of infection(s).

SUMMARY

Because management of BT and BW events depends on the disease surveillance, laboratory, and outbreak investigation capabilities of public health authorities, the science of epidemiology will always be the foundation for a response to these events. An enhanced index of suspicion, awareness of potential red flags, open lines of communication between local healthcare providers and law enforcement authorities, knowledge of historical outbreak investigation information, robust disease surveillance systems, and the use of advanced molecular techniques will improve the ability to respond to any future BT or BW event.

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Chapter 3

FOOD, WATERBORNE, AND AGRICULTURAL DISEASES

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SUMMARY

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INTRODUCTION

Food and waterborne pathogens cause a considerable amount of disease in the United States. The economic impact from foodborne diseases is estimated at about $78 billion per year.[1] The top five pathogens that contribute to domestic foodborne illness are, Novovirus, *Salmonella* species, *Clostridium perfringens*, *Campylobacter* species, and *Staphylococcus aureus*.[2,3] Many of the common foodborne pathogens, whether bacteria, viruses, parasites, or toxins, can cause disease if purposefully introduced into water or food sources. These pathogens characteristically have the potential to cause significant morbidity or mortality, have low infective dose and high virulence, are universally available, and can be stable in food products or potable water. These agents include *Clostridium botulinum* toxin, the hepatitis A virus, *Salmonella*, *Shigella*, enterohemorrhagic *Escherichia coli* species, *Cryptosporidium parvum*, *Campylobacter jejuni*, *Listeria monocytogenes*, and *Vibrio cholerae*, among others. Pathogens in the Centers for Disease Control and Prevention (CDC) list of biological threat agents that also may cause food or waterborne disease are *Bacillus anthracis*, *Brucella* species, staphylococcal enterotoxin B, and ricin.[4] The potential for nonlisted biological agents such as mycotoxins and parasites (eg, *Taenia* species) to be used in a bioterrorist event should also be considered.

This chapter provides an introduction to the far-reaching subjects of food and waterborne diseases, the potential for terrorist attacks on the food and water supply, and terrorism directed at sources of the nation's farm-to-food continuum (agricultural terrorism). For a more extensive review of these topics, readers may consult more specialized texts on food[5] and waterborne[6] diseases and agricultural terrorism.[7,8]

FOODBORNE AND WATERBORNE PATHOGENS AND DISEASES

Bacillus anthracis

B anthracis is the causative agent of two forms of foodborne anthrax: oropharyngeal and gastrointestinal. Although spores of *B anthracis* would cause the most potential harm via an aerosol release, anthrax disease is not normally perceived as having bioterrorism potential as a foodborne bacterial contaminant because the infective dose required for such an attack would be high.[9] However, given that the early diagnosis of gastrointestinal anthrax is difficult for clinicians who have never treated cases of this disease, a higher mortality rate than expected may result from a natural or purposeful outbreak. Anthrax spores are resistant to disinfection by contact chlorination as used by water treatment facilities, although higher levels of chlorination (≥ 100 ppm) for longer contact times (5 minutes) will kill *Bacillus* spores.[10]

Clostridium botulinum

C botulinum is the causative agent of botulism intoxication, of which there are three natural manifestations: classic, wound, and infant botulism. Bioterrorism use of botulinum toxin could possibly occur through inhalational intoxication, as was considered by the Aum Shinrikyo cult in Japan in 1990.[11,12] *C botulinum* produces the most potent natural toxin known; the human lethal dose of type A toxin is approximately 1.0 μg/kg.[13] There are seven antigenic types of botulinum toxin, denoted by the letters A through G. Most human disease is caused by types A, B, and E. Botulinum toxins A and B are often associated with home food preparation[14] and home canning[15] and pickling.[16] Botulism-contaminated food cannot be distinguished by visual examination, and the cook is often the first to show the toxin's effects (via sampling the food during cooking). A 12- to 36-hour incubation period is common. The incubation period is followed by blurred vision, speech and swallowing difficulties, and descending flaccid paralysis.[17]

The current mortality rate associated with botulism intoxication is less than 10%. Foodborne botulism mortality during the 1950s (before the advent of modern clinical therapies) was approximately 25%.[18] Little evidence of acquired immunity from botulinum intoxication exists, even after a severe infection. Successful treatment consists of aggressive trivalent (A, B, E) botulinum antitoxin therapy and ventilatory support. Early diagnosis is critical for patient survival. Toxin can be found in food, stool, and serum samples, which may all be used in the standard mouse model assay to test for the presence of botulism toxin.[19]

A controversial paper published in 2005[20] explored the potential for botulinum toxin contamination of the milk supply. A nine-stage dairy cows-to-consumer supply chain was examined, which accurately modeled a single milk-processing facility. The release of botulinum toxin was assumed to have occurred either at a holding tank at the dairy farm, in a tanker truck transporting milk from the farm to the processing plant, or at a raw milk silo at the plant. By the use of this model, it was predicted that 100,000 individuals could be poisoned with over 1 gram of toxin, and 10 grams

would affect about 568,000 milk consumers.[20] The National Academy of Sciences published this information to foster further discussion and alert authorities to dangers to the milk supply from purposeful contamination.[21] The paper also describes interventions that the government and the dairy industry could take to prevent this scenario. Officials at the US Department of Health and Human Services requested that the paper not be published, but the National Academy of Sciences published it anyway, convinced that the information would not enable bioterrorists to conduct an attack, and that the paper would stimulate biodefense efforts. Whether the paper's information presents a "roadmap for terrorists" by exposing vulnerabilities in food processing remains to be determined[22]; however, the hypothetical use of botulinum toxin placed at various points into the food supply was proposed in a fictional novel over 35 years ago.[23]

Campylobacter jejuni

Campylobacter, *Salmonella*, *Listeria*, and *E coli* O157:H7 can be transmitted zoonotically from contaminated animal food sources. These bacteria species are ubiquitous and cannot be completely eliminated from the food supply. *C jejuni* is the most commonly reported bacterial cause of foodborne infection in the United States.[24] Chronic sequelae associated with *C jejuni* infections include Guillain-Barre syndrome[25] and arthritis.[26] Infants have the highest age-specific isolation rate for this pathogen in the United States, which is attributed to a greater susceptibility upon initial exposure and a lower threshold of seeking medical treatment for infants.[24] Reservoirs for *C jejuni* include wild fowl and rodents.[27] The intestines of poultry are easily colonized with *C jejuni*,[28] and it is a commensal inhabitant of the intestinal tract of cattle.[29] Antibiotic resistance of *Campylobacter* is a growing concern for poultry farmers.[30] The infective dose for *Campylobacter* is 100 to 1,000 cells, with poultry the primary source of infection in the United States.[31] Insect transmission by several fly species has also been documented.[32] There is a 3- to 5-day illness onset for campylobacteriosis and a 1-week recovery time. Immunity is conferred upon recovery, which accounts for a significantly higher incidence rate among individuals younger than 2 years of age in developing countries.[33]

Salmonella

Salmonellosis is the second most common foodborne illness,[34] and contaminated food is the principal route of disease transmission.[35] There are over 2,400 *Salmonella* serotypes, many of which can cause gastroenteritis, manifested as diarrhea, abdominal pain, vomiting, fever, chills, headache, and dehydration.[34,35] Other diseases from *Salmonella* infections include enteric fever, septicemia, and localized infections. Poultry is a principal reservoir of the salmonellae.[35] Water, shellfish, raw salads, and milk are also commonly implicated as vehicles for this pathogen. In humans, the most highly pathogenic *Salmonella* species is *S typhi*.[35] This bacterium is the causative agent of typhoid fever, which comprises about 2.5% of salmonellosis in the United States.[35] The symptoms of typhoid include septicemia, high fever, headache, and gastrointestinal illness.[35]

During World War II, the Japanese developed biological weapons, poisoning prisoners with *S typhimurium* and many other bacteria and viruses during their experimentation, and contaminating wells with *S typhimurium* along the Russian border of Mongolia.[36] In September and October 1984, two large groups of salmonellosis cases occurred in The Dalles, Oregon. Case interviews by health officials associated patronage of two restaurants in The Dalles with illness, especially with food items eaten from salad bars. *S typhimurium* isolates were then obtained from clinical specimens.[37] The size and nature of this outbreak helped to initiate a criminal investigation, which was rarely done in conjunction with a foodborne disease outbreak. The cause of the epidemic became known when the Federal Bureau of Investigation investigated a nearby cult (the Rajneeshees) for additional criminal violations.[38] In October 1985 authorities found an opened vial that contained the original culture type of *S typhimurium* inside a refrigerator within the Rajneeshee clinic laboratory.

A large multistate outbreak of milk-borne salmonellosis from *Salmonella enteritica* serovar *typhimurium* occurred in northern Illinois in 1985, with more than 14,000 people reported ill and five deaths.[39,40] The cause of the outbreak was the accidental comingling of raw milk into the pasteurized product in a milk plant.[41] The contaminated milk was distributed via supermarket distribution systems, and cases were also reported in the neighboring states of Indiana, Iowa, and Michigan.[42] Medical treatment was complicated because the strain of *S typhimurium* involved was found to be resistant to antibiotics. Such inadvertent milk-borne contamination reinforces the potential for a ready-made vehicle for transmission of disease among a population by deliberate means.[20]

Listeria monocytogenes

L monocytogenes is ubiquitous in the environment and often found in silage, water, and the environs of animal fodder.[43] Soft cheeses,[44] raw or contaminated

milk,[45] and contaminated refrigerated foods[46] are often sources of this organism. Listeriosis can result in meningo-encephalitis and septicemia in neonates and adults, and fever and abortion in pregnant women.[47] Fetuses, newborns,[48] the elderly,[49] and immunocompromised persons[50] are at greatest risk for serious illness. Listeriosis case investigations can be problematic because of the variable incubation period for illness (3 to >90 days). Large outbreaks of foodborne listeriosis have occurred, including a 1983 Massachusetts epidemic where improperly pasteurized milk was the source of the infection.[51] Of the 49 infections associated with this outbreak, 14 patients died.[51]

Escherichia coli

E coli O157:H7 infections often originate from contamination due to a bovine reservoir.[52] This organism produces two verotoxins and is a significant cause of serious pediatric illness.[53] It can result in bloody diarrhea and hemolytic uremic syndrome, which is defined as the demonstration of three clinical conditions: (1) microangiopathic hemolytic anemia, (2) acute renal failure, and (3) thrombocytopenia.[53] Children younger than 5 are at greatest risk for hemolytic uremic syndrome when infected with *E coli* O157:H7 or other enterohemorrhagic *E coli* (EHEC) species, and deaths from these infections occur most often in the age ranges of 1 to 4 years and 61 to 91 years.[52] A major source of EHEC exposure is from consumption of and contact with beef cattle.[54] About 20% of the ground beef consumed in the United States is derived from culled dairy cattle, which may be an important contributor to this bacterial contamination of the food supply.[55] For example, during July 2002, the Colorado Department of Public Health and Environment identified an outbreak of *E coli* O157:H7 infections that linked 28 illnesses in Colorado and six other states to the consumption of contaminated ground beef products. Seven patients were hospitalized, and five developed hemolytic uremic syndrome.[56]

E coli-contaminated food items commonly result from use of cattle waste as fertilizer or other contact with cattle products. Outbreaks have occurred from exposure to various *E coli*-tainted food items, including alfalfa[57] and radish[58] sprouts, parsley,[59] lettuce,[60,61] hazlenuts,[62] apple cider,[63] unpasteurized gouda cheese,[64,65] raw milk,[66] recontaminated pasteurized milk,[67] prepackaged cookie dough,[68] and salami,[69] as well as through petting zoos[70] and environmental transmission.[71,72] Waterborne outbreaks of *E coli* O157:H7 have also occurred. From mid-December 1989 to mid-January 1990, 243 cases of gastrointestinal illness from antibiotic-resistant *E coli* O157:H7 occurred in a rural Missouri township as a result of an unchlorinated water supply.[73] Swimming-associated outbreaks of *E coli* O157:H7 have also occurred.[74,75]

Other enterohemorrhagic *E coli* strains containing Shiga toxins (Shiga toxin-producing *E coli* [STEC] infections) have appeared as public health concerns, including STEC O121,[76] STEC O26,[77] STEC O145,[78] and STEC O104:H4.[79] Novel STEC strains can easily develop due to opportunistic microbial growth and spread in the food supply chain and distribution systems. The CDC *E coli* investigation page[80] lists current and past identified outbreaks. A recent 10-year study in Connecticut of 663 reported STEC infections demonstrated that both O157 and non-O157 STEC infection incidence decreased from 2000 through 2009, and also that O157 was the most common and clinically severe type of STEC infection.[81] However, in this and other studies, non-*E coli* O157 accounted for a minority of all clinically significant STEC infections.[82,83] Importantly, STEC O104 and O157:H7 infections are more likely to lead to hospitalization and death than other STEC serogroups, as shown by a recent 8-year retrospective cohort study of 8,400 patients in Germany.[84] Another recent German study demonstrated that cattle density is a risk for exposure to *E coli* O157:H7 and other STEC strains, including all major disease-causing groups (O26, O103, O111, O128, O145), but not O91.[85] STEC strains therefore appear to be a diverse group of organisms that demonstrate differences as well as many commonalities in exposure and epidemiology.

Shigella

Humans are the major reservoir for *Shigella* and the primary source of subsequent infections.[86] It is thought that worldwide *Shigella*-associated illness causes about 165 million cases per year, of which fewer than 1% occur in industrialized nations.[86] *Shigella dysenteriae* produces severe disease, may be associated with life-threatening complications, and causes about 25,000 cases of illness each year in the United States.[86] Although not an environmentally hardy organism, *Shigella* is highly infectious and can be very persistent in a close community environment.[87] Four serogroups (A through D) cause approximately 80% of shigellosis cases in the United States. Immunity is serotype-specific.[88] Vaccine development has been problematic,[89] and the species can easily become resistant to antibiotics.[90] Infants and young children are most susceptible to shigellosis, attributable in part to toiletry behaviors and child care practices. The infectious dose for *Shigella* is 10 to 100 organisms, and *Shigella* contamination can cause outbreaks associated with food, water, and milk. Shigellosis has also been associated with recreational

swimming.[91] Shigellosis is readily transferred from person-to-person contact and through fomites[92]; it can also be transmitted by insect vectors (primarily flies).[93] There is a 1- to 3-day incubation period for shigellosis. *Shigella* organisms are shed for 3 to 5 weeks after symptoms cease, ultimately contributing to a greater person-to-person spread than with other enteric pathogens such as *Salmonella* and *V cholerae*.

Cryptosporidium

Cryptosporidium, a protozoan and an obligate intracellular parasite, can cause food and waterborne illness and can also be acquired from exposure to contaminated recreational water.[94–98] Seroprevalence surveys indicate that about 20% of the US population have been infected with *Cryptosporidium* by adulthood.[99] The severity and course of infection can vary considerably, dependent upon the immune status of the individual. Intestinal cryptosporidiosis is often characterized by severe watery diarrhea but may also be asymptomatic. Pulmonary and tracheal cryptosporidiosis in humans is associated with coughing and low-grade fever; these symptoms are often accompanied by severe intestinal distress. The duration of illness in one study of 50 healthy individuals varied from 2 to 26 days, with a mean of 12 days.[100]

The precise infectious dose is unknown; research indicates that a range of 9 to 1,024 oocysts will initiate infection.[101] The pathobiology is not completely known either; however, the intracellular stages of the parasite can cause severe tissue alteration. Infected food handlers are a major contributor to disease transmission. Consequently, cryptosporidiosis incidence is higher in facilities that serve uncooked foods, such as restaurants with salad bars. Child care centers can be a problematic source of cryptosporidium infection because diarrhea in children in diapers can be difficult to contain.[102] A significant reservoir worldwide for *Cryptosporidium parvum* is domestic livestock, predominately cattle.[103] Drinking water outbreaks have affected as many as 403,000 individuals (in a 1993 outbreak in Milwaukee).[94] In the Milwaukee incident, the water was both filtered and chlorinated.[104] The organism's resistance to chlorine treatment ensures that it will remain a concern in treated potable water,[105] and therefore a risk to immunocompromised individuals, in whom it causes severe and chronic life-threatening gastroenteritis.[106]

Hepatitis A

Humans are the source of the *Hepatovirus* hepatitis A virus.[35] Illness caused by hepatitis A is characterized by sudden onset of fever, malaise, nausea, anorexia, and abdominal discomfort, followed by jaundice.[35] The infectious dose is not precisely known but is thought to be 10 to 100 virus particles.[35] The virus is hardy, and it survives on hands and fomites. Because viral particles are excreted in the feces during clinical illness, stringent personal hygiene is crucial to prevent disease transmission. Hepatitis A is commonly transmitted via personal contact, and fewer than 5% of all hepatitis A cases are demonstrated to have been caused by food or waterborne transmission.[107] Permanent immunity to hepatitis A is assumed subsequent to infection[108] or immunization completion.[109] The advent of nationwide hepatitis A vaccination programs is gradually causing a decrease in disease incidence and the susceptible population.[110] As a result, hepatitis A may in time cease to be a public health concern.[111]

The potential for hepatitis A virus transmission in drinking water was demonstrated in an outbreak among members of the varsity football team at the College of the Holy Cross in Worcester, Massachusetts, in 1969. The same water supply was used for both irrigation and potable water. Water used by firefighters to battle a blaze nearby caused a drop in water pressure, and back-siphonage brought groundwater into the football practice field's irrigation system. The groundwater had been contaminated by children infected with hepatitis A in a building immediately adjacent to the playing field. The football team members became ill after consuming the water from a faucet hooked up to this contaminated water source.[112,113] Although 90 of 97 players and coaches on the team became ill (93% attack rate), serologic testing performed years later revealed that only 33 had IgM anti-hepatitis A virus in serum (34% attack rate).[114] Because of this discrepancy, the illness may have been caused by another pathogen also present in the water.

Mycotoxins

Fungi are plant pathogens that can cause both mycoses (infections) and mycotoxicoses (exposures to toxic fungal metabolites that may be dietary, dermal, or respiratory). Mycotoxins are ubiquitous worldwide toxic fungal metabolites and contaminants of stored cereal grains.[115,116] Although mycotoxins are not on the CDC threat list, individuals with chronic exposure to mycotoxins (including aflatoxin B1, ochratoxin, T-2 toxin, deoxynivalenol [DON], nivalenol [NIV], and others), often exhibit oncogenic symptoms, including liver damage, liver cancer, hemorrhaging, mental impairment, abdominal pain, vomiting, convulsions, and edema. The fact that these toxins are found naturally in commercially available cereal-based foods, including bread and related products, noodles, breakfast cereals, baby and infant foods, and rice, indicate

that a ready substrate for growth is available, and deliberate contamination of these foodstuffs may be possible. Mycotoxicoses are often undiagnosed and hence unrecognized by public health authorities, except when large numbers of people are affected.[117] The symptoms of mycotoxicosis depend on the type of mycotoxin; the amount and duration of exposure; the age, health, and sex of the exposed individual; and many unknown synergistic effects including genetics, dietary status, and interactions with other toxic insults.[118]

Large naturally occurring outbreaks of trichothecene intoxications have occurred, including a large exposure of trichothecene mycotoxin from moldy grain and bread in Orenburg, Russia, in 1944, which caused alimentary toxic aleukia and subsequent mortality in at least 10% of the population.[119] A 1991 outbreak caused by moldy wheat and barley affected 130,000 people in the Anhui province in China.[115] Fusarium mycotoxins including DON and NIV have also been discovered in corn samples in Linxian, China, in positive correlation with the incidence of esophageal cancer.[119,120] Although outbreaks of mycotoxicoses have decreased greatly as a result of increases in hygiene measures, they still occur in developing countries,[121] are considered a serious international health problem,[122] and also pose a risk to domestic animals.[122–126]

The history of mycotoxin use as a biological weapon includes efforts by Iraq to develop and use aflatoxins during the 1980s.[127,128] Iraq's biological weapons program cultured strains of *Aspergillus flavus* and *A parasiticus* and extracted 2,300 liters of concentrated toxin.[127,128] This aflatoxin was used mostly to fill missile warheads, and the remainder was kept stockpiled.[127,128] The Soviet Union is suspected of deploying trichothecene toxins (NIV, DON, and T-2) in the "yellow rain" incidents in Laos and Cambodia during the 1980s. Whether the toxin exposures that occurred at that time were the result of purposeful[129] or natural[130] events has never been completely resolved. These events indicate the potential for mycotoxin use as a biological weapon or bioterrorism agent.

Parasites

Parasites such as tapeworms (eg, *Taenia* species) may have potential for use as bioterrorism agents. It is conceivable, for example, that a culture of *Taenia solium* eggs could be poured onto a salad bar or into water, be ingested, and cause illness. Symptoms of

TABLE 3-1

PROPERTIES RELATED TO THREAT POTENTIAL OF COMMON FOOD AND WATERBORNE DISEASE PATHOGENS

Pathogen	Incubation Period	Infective or Toxic Dose*	Mortality in United States	Bloody Diarrhea
Enterohemorrhagic *Escherichia coli*	3–4 d	$10–10^2$	rare	yes
Salmonella typhi	8–14 d	$10–10^2$	low	yes
Salmonella species	6–72 h	$10^2–10^3$	low	yes
Shigella dysenteriae	1–7 d	$10–10^2$	rare	yes
Campylobacter jejuni	2–5 d	$\geq 5 \times 10^2$	rare	no
Clostridium botulinum toxin	12–72 h	70 μg†	5%–10%	no
Vibrio cholera	2–3 d	10^6	rare	no
Cryptosporidium species	7 d	9–1,024	rare	no
Listeria monocytogenes	3 ≥ 90 d	unknown	high	no
Hepatovirus hepatitis A	30 d	$10–10^2$	low	no
Norovirus	1–2 d	$< 10^2$	rare	no
Mycotoxins	Minutes to months‡	4 mg/kg§	rare	yes

*The number of organisms unless otherwise noted.
†Oral lactate dehydrogenase for a 70-kg human.
‡Dose-dependent.
§Oral lactate dehydrogenase for laboratory rat.

taeniasis from ingestion of the eggs would include cysticercosis (parasite tissue infection), which would not appear for weeks to years following infection. However, this infection timeline should not eliminate parasites from consideration as potential bioterrorism agents; such a scenario has been proposed.[131] *T solium* can be transmitted person to person by food handlers with poor personal hygiene, adding to the spread of the outbreak.[132] Such an outbreak may go undiagnosed for an additional period, during which ill persons are seen by healthcare providers unfamiliar with tapeworm infections. A purposeful outbreak of giardiasis that occurred in Edinburgh, Scotland, in 1990 demonstrates that parasites can be used for bioterrorism. Nine individuals living in the same apartment complex developed giardiasis subsequent to the purposeful fecal contamination of an unsecured water supply.[133]

Threat Potential Summary

Table 3-1 provides information about various pathogens related to their potential threat as purposeful food contaminants. Both bacterial and viral enteric pathogens were considered for this compilation. This taxonomic approach may prove useful in stimulating further discussion of pathogenicity and potential for misuse. For example, *Salmonella* was not considered a threat agent before its use in the salad bar contamination in 1984. The prior view may have been based on factors inherent in *Salmonella* infection—a high dose of *Salmonella* is required to cause illness. If the infectious or toxic dose required for illness from an organism is the sole consideration for its classification as a bioweapon, then salmonellae should not even be considered as a threat agent. However, the use of *S typhimurium* to sicken many hundreds of people in the Dalles incident demonstrated a reality of biological agents: those that can be cultured and dispersed to cause illness will prove effective. Although no deaths occurred, the incident involved a rapid-onset illness with gastrointestinal effects that spread through 10 restaurants, causing widespread fear of food poisoning and long-lasting economic consequences in the community.[134] Given suitable circumstances, almost any pathogen could be used to make a target population ill. The severity of illness, including symptoms such as bloody diarrhea, also should be considered. For example, an outbreak of bloody diarrhea could have strong psychological effects upon those directly affected and perhaps lead to widespread psychological effects in the general public[135] if exacerbated by media coverage of the outbreak.[136]

WATER SUPPLY CONCERNS

Poisoning water supplies is one of the oldest methods of biological warfare.[137] The earliest documented poisoned drinking water occurred in Greece in 590 BCE, when the Amphictyonic League used hellebore to poison the city of Kirrha's water source, causing the inhabitants to become violently sick and unable to move.[138] In current developed countries, it is more difficult for a terrorist to contaminate water because of the large volumes of water and the extensive purification processes used in modern water treatment facilities, including aeration, coagulation and flocculation, clarification, filtration, and chlorination.[138] All of these methods remove contaminants and pathogens in the water, whether purposefully added or not.

However, the risk to the US water supply has been known for some time. Federal Bureau of Investigation Director J Edgar Hoover noted in 1941, "It has long been recognized that among public utilities, water supply facilities offer a particularly vulnerable point of attack to the foreign agent."[139] A terrorist might bypass the purification process and introduce a pathogen later in the distribution system. A private well water supply system with a smaller volume of water and a less extensive purification system may be more vulnerable. Another potential avenue for deliberate waterborne contamination is the addition of a pathogen to a building's water supply (ie, an enclosed system), with likely little or no subsequent water treatment processes and a specific target community.

Waterborne pathogens included on the CDC threat list are *V cholerae* and *C parvum.* The Milwaukee outbreak of *C parvum* demonstrates the potential of public water supply contamination to affect great numbers of people. Another example of an extensive waterborne disease outbreak resulting from contaminated well water was the 1999 *E coli* O157:H7 and *Campylobacter* outbreak involving more than 900 illnesses and 2 deaths among attendees of a New York county fair.[140] According to a comprehensive review of potable water threats by Burrows and Renner, potential water threat agents also include *B anthracis, Brucella, V cholera, C perfringens*, *Yersinia pestis*, *Chlamydia psittaci*, *Coxiella burnetii*, *Salmonella*, *Shigella*, *Francisella tularensis*, enteric viruses, smallpox virus, aflatoxin, *C botulinum* toxin, microcystins, ricin, saxitoxin, staphylococcal enterotoxins, T-2 mycotoxin, and tetradotoxin.[141] The 1969 hepatitis A outbreak at the College of the Holy Cross demonstrates the potential for this pathogen to cause illness when distributed in a water supply.

Communitywide outbreaks of gastroenteritis, caused by *Giardia lamblia*, *Cryptosporidium*, various *E coli* serotypes, *Torovirus*, and other infectious agents, have occurred from recreational water use, including swimming pools, water slides, and wave pools.[142] Nongastroenteritis recreational water outbreaks often include those caused by *Pseudomonas aeruginosa*, *Naegleria fowleri*, and *Legionella*.[142] A recent naturally occurring outbreak of gastroenteritis associated with a contaminated recreational water fountain at a Florida beachside park demonstrates the potential for disease transmission.[143] In this incident, 44% of the interviewed park visitors who used an interactive water fountain became ill. Both *C parvum* and *Shigella sonnei* were subsequently isolated from clinical specimens obtained from these ill persons. The median age of the ill persons was 8 years. One can imagine the effect of a powerful biological agent such as *C botulinum* toxin covertly added to a recreational public water fountain in similar circumstances.[144]

The water utility industry and federal public health agencies have carried out plans to improve the ability to prevent as well as detect deliberate contamination of water systems.[145] An example of a new program to detect purposeful contamination of the water supply is the Water Sentinel program.[146] However, much work remains to attain full biosecurity of the US water supply.[147,148]

AGRICULTURAL TERRORISM

Agricultural terrorism (agroterrorism) may be directed at stored or processed food, but some of the greatest vulnerabilities may exist close to the farm end of the farm-to-food continuum (Figure 3-1). Many of the potential bioterrorist agents are endemic, and therefore cannot easily be controlled. As with processed food and water terrorism, agroterrorism concerns are not recent developments. The historical use of biological agents to affect livestock includes the attempt to interrupt supply lines by infecting cavalry and transport animals with anthrax and glanders during World War I. In April 1915, German-American physician Anton Dilger (who had served in the German Army) returned to the United States from Germany along with cultures of *Burkholderia mallei* and *B anthracis*. His intent was to infect animals (horses and mules) that were shipped from the United States to France and England for use in cavalry and transport to support their war with Germany. Dilger propagated the bacterial cultures and tested them for virulence using guinea pigs in the basement of a house (known as "Tony's Lab") he and his brother Carl rented in Chevy Chase, Maryland, near Washington, DC.[149] Over the next 2 years, Dilger's bacterial cultures were used to infect horses and mules in holding pens in docks at the ports of Baltimore, Maryland; Newport News, Virginia; Norfolk, Virginia; and in New York City. Stevedores working for German steamships were recruited and provided with cork-stoppered glass vials containing the bacterial cultures, in which a hollow steel needle had been placed. The stevedores were instructed to wear rubber gloves while jabbing the animals with the needles. These cultures were also spread among the animals by pouring them directly into the animal feed and drinking water.[150,151]

The significance of foot and mouth disease (FMD) as a biological weapon has been known for some time, and it is perhaps the greatest agroterrorism threat for livestock. Field trials of FMD virus dissemination were conducted in Nazi Germany's offensive biological warfare program. FMD is thought to be inherently spread through airborne virus transmission, a problematic



Figure 3-1. Some of the greatest vulnerabilities from agricultural terrorism may exist at the farm end of the farm-to-food continuum.
Photograph courtesy of the US Department of Agriculture, Washington, DC.

issue for outbreak containment,[152] and Germany considered aerial dissemination and dispersal of the FMD virus through contaminated hay and grass.[153(p114)]

Another attack on livestock occurred during the Mau-Mau uprising in British-controlled Kenya (1952–1960), when the Mau-Mau used the indigenous poisonous African milk bush (*Synadenium compactum*) to kill 33 cows at a mission station in 1952.[154,155] This use of locally obtained poisonous plants could be replicated anywhere lacking constant monitoring of animal feed.

Anticrop terrorism has also been suspected on numerous occasions. The Colorado potato beetle (*Leptinotarsa decemlineata*) is a crop pest of plants of the genus *Solanum*, which includes potatoes, tomatoes, and eggplants. During World War II, Germany initiated large-scale breeding and field trial dispersals of the insects in Germany, when Dr Martin Schwartz conducted an offensive research program at the Kruft Potato Beetle Research Station near Koblenz.[153(p110)] This program may have backfired by initiating local crop infestations; however, outbreaks of the pest also occurred in England and the United States, which were suspected to be caused by a German release of the insects.[156,157] Perhaps because of this research conducted in Nazi Germany, in 1950 Soviet-occupied East Germany accused the United States of releasing the beetle during in infestation.[158] Herbicides have also been used for wartime missions, such as the large-scale use of the defoliant Agent Orange by the United States to both defoliate and destroy crops used by North Vietnamese forces.[159] In 1989 a group known as "the Breeders" announced that it had released Mediterranean fruit flies in southern California to protest the use of pesticides in that region.[160]

State-sponsored agricultural terrorism remains a global concern today, given vulnerabilities inherent in modern farming practices. In the United States, livestock may be susceptible to agroterrorism (Figure 3-2). Because US disease eradication efforts among livestock herds have been so successful, much of the nation's livestock is either vaccinated or monitored for disease by farmers and veterinarians. However, the risk of harm from agroterrorism to large numbers of livestock has been increased through the widespread use of modern livestock farming, such as concentrated animal feeding operations (CAFOs). The pervasiveness of CAFOs in the US agriculture industry is all-encompassing. For example, in the US between 1997 and 2007:[161]

- hog factory farms added 4,600 hogs every day;
- factory farm dairies added nearly 650 cows every day;
- factory farms added 5,800 broiler chickens every hour;



Figure 3-2. Livestock may be more susceptible to agroterrorism than crops.
Photograph courtesy of the US Department of Agriculture, Washington, DC.

- factory farmed broiler chickens doubled to 1.1 billion;
- hog factory farms grew by 42%, to 5,144;
- the average size of egg factory farms increased by half to 614,000 hens;
- the number of cows on factory-farm dairies nearly doubled, to 4.9 million;
- the number of hogs on factory farms grew by more than a third, to 62.9 million;
- the number of factory farm egg-laying hens increased by 24%, to 266.5 million;
- the number of US beef cattle on industrial feedlots grew by 17%, to 13.5 million; and
- nearly half of factory-farm egg-laying hens were located in just five states: Iowa, Ohio, Indiana, California, and Pennsylvania.

Altogether, CAFO aggregation has had the greatest effect on livestock operations with the greatest numbers of animals. From 1982 to 1997, livestock operations with 1,000 or more animals increased by 47%. In comparison, farms with less than 25 animals (a family farm) decreased by 28%.[162] While such aggregation has enabled economic viability and success for the farming industry, it also can provide a single-source opportunity for foodborne contamination or adulteration for the would-be bioterrorist desiring to affect the food supply.

Upon infection, livestock may become a vector[163] or reservoir[164] for disease transmission. This potential was plainly demonstrated in the 2001 outbreak of

FMD in the United Kingdom.[165] This outbreak was the single largest FMD epidemic ever experienced in the world.[166] Agricultural and food losses to the United Kingdom exceeded $4.6 billion,[167] and psychological effects in residents of the worst affected areas were extensive and long-lasting.[168] The United States has not had an outbreak of this disease since 1929,[169] and the US Department of Agriculture (USDA) has developed national protective measures to prevent a reintroduction.[170]

Perhaps the greatest national risks from agroterrorism involve the potential for widespread economic consequences. Not only would immediate loss to a crop occur from such an event, but incidental costs would also result from lost production, the destruction of potentially diseased products, and containment (including quarantine, drugs, and diagnostic and veterinary services). The costs of these programs would be borne by farmers as well as federal and state governments.[171] Export markets would be rapidly lost as other nations close their borders to imports from a country with diseased livestock. As an example, a single case of mad cow disease (bovine spongiform encephalopathy) was found in Washington state on December 23, 2003; by December 26, Japan had banned all US beef imports, and beef prices dropped by as much as 20% in the following week.[172] Additionally, multiplier economic effects would occur from decreased sales by agriculturally dependent businesses and tourism. Other animal pathogens besides FMD and bovine spongiform encephalopathy that could have severe economic consequences if uncontrolled include highly pathogenic avian influenza,[173] rinderpest,[174] and African[175] and classical swine fever.[176]

The USDA's Animal and Plant Health Inspection Service has developed a select agent and toxin list of pathogens and toxins that endanger agriculture in the United States[177] (some of these zoonotic pathogens also endanger humans and appear on the CDC Category A list[4]; these pathogens are listed separately by the USDA as overlap agents and toxins). Another USDA list enumerates harmful plant pathogens.[177]

SMUGGLING AND INVASIVE SPECIES

The problem of smuggling and the unintentional introduction of invasive species has grown in recent years. Increased and more rapid international trade, increased trade in fresh commodities, new travel and trading routes, and increased difficulties in enforcing quarantines have all contributed to this problem. The potential consequences of smuggling and unintentional introduction of invasive species may go far beyond the direct damages or costs of control. The economic costs of all invasive species in the United States is estimated at between $120 billion and $138 billion per year.[178,179] These invasive species consist of microbes (30.1%), mammals (27.2%), plants (25.0%), and arthropods (15.4%). The full range of economic costs of biological species invasions reaches far beyond the immediate impacts on the affected producers and often include consequences to local, national and global markets. In 1995, member nations of the World Trade Organization signed the Uruguay Round Agreement on the Application of Sanitary and Phytosanitary Measures, which set out basic rules for food safety and animal and plant health standards and increased the transparency of sanitary and phytosanitary measures.[180] The agreement covers all measures to protect human or animal health from foodborne risks, human health from animal- or plant-carried diseases, and animals and plants from pests or diseases.[180]

FOOD AND WATER SECURITY

On December 3, 2004, the former secretary of the Department of Health and Human Services, Tommy Thompson, warned of a possible terrorist attack on the nation's food supply: "For the life of me, I cannot understand why the terrorists have not attacked our food supply, because it is so easy to do . . . We are importing a lot of food from the Middle East, and it would be easy to tamper with that."[182] In American society, the farm-to-food continuum, which includes production, processing, distribution, and preparation, has myriad potential vulnerabilities for natural and intentional contamination.[182] Centralized food production and widened product distribution systems present increased opportunities for the intentional contamination of food.[183] As covered in the discussions above, many opportunities exist along the food and water production continuum to accidentally or intentionally introduce various pathogens, many of which are not categorized as threat agents.[184] Strategies to counter these threats should focus on enhancing knowledge of all raw material inputs to the system; identifying and addressing the most likely points of vulnerability; disposing of end products after they leave the systems; and accounting for employees, visitors, computers, and physical security throughout the continuum.

Knowledge of the various processes involved in food production will help to determine potential vulnerabilities for agricultural terrorism. The typical food distribution system includes agricultural production and harvesting, storage and transport of raw commodities, processing and manufacture, storage and transport of processed and manufactured products, wholesale and retail distribution, and the food service sector.[185] The responsibility for food safety and security throughout the food distribution network is shared by the producers and suppliers as well as many different state and federal agencies. Typically, a state's health and agricultural agencies ensure that the food comes from safe sources and is served with safeguards to prevent foodborne disease transmission. Equivalent federal agencies share these responsibilities, including the US Food and Drug Administration, USDA, Department of Health and Human Services, US Public Health Service, CDC, and other partner agencies now part of the Department of Homeland Security, including the Federal Bureau of Investigation and the US Customs Service.

One prevention strategy is to anticipate intentions or motivations that could result in an attack using a particular product or organization. These motivations could include religion or ideology; personal grievances (real or perceived); and contentious issues such as animal rights, environmental protection, and abortion. Research facilities, food processors, and food retailers could be targets of terrorism and should take extra preventive measures. Knowledge of terrorism trends can be an indicator for the need to change security measures to meet the threat. However, because the US food industry is highly competitive on a price basis, additional preventive measures may only be an option if they are government subsidized.

From an attacker's standpoint, the choice of methods and weapons is determined by the target and the delivery medium. It is rare that someone would attempt to cause harm without consideration of whom or how many people are affected. The target population may then define the vulnerabilities. For example, animal feed could be contaminated if the goal was to affect a CAFO.

Strategies also can be implemented to address specific vulnerabilities. The first task is to define production processes in terms of the inputs and outputs at all potential nodes of vulnerability. For example, foods that are either eaten uncooked or that can be contaminated after cooking should receive special quality control attention. Also, knowledge of where raw materials including water are obtained can help identify needs for enhanced security and accountability.

A thorough knowledge of the existing hazard analysis critical control points (HACCPs) for each food item considered to be a potential vehicle for foodborne disease is essential to understanding and preventing illness. A comprehensive HACCP analysis will provide a systematic method of documenting that food safety hazards have been addressed.[186] Hazard analysis involves food safety issues only, including storage and holding temperatures, pH, sanitary conditions, physical storage security, and any other factor that could impact the safety and integrity of a food item during manufacture, storage, delivery, or food preparation.[186] General guidance to conducting an HACCP program would necessarily include:[186]

- Hazard analysis: what are the food safety hazards that can be controlled?
- Establish critical control points (CCP): where can things go wrong, and how can they be controlled?
- Establish critical limits: what physical values (temperature, pH, etc) indicate that the process is in control?
- Establish monitoring procedures: how will the CCPs be monitored?
- Establish corrective actions: what happens if a critical limit is exceeded?
- Establish a record keeping system: "If it isn't written down, it didn't occur."
- Establish verification procedures: how can you know if the system works?

HACCP principles have been successfully applied to the production, storage, and serving of many types of food items.[187–189] However, there remain many challenges to providing a safe and wholesome food supply, and resolution of issues through the use of HACCP may also provide solutions that could prevent bioterrorism. For example, food items that are common sources of foodborne infections may also present opportunities to a potential bioterrorist, by virtue of a lack of proper temperature use and monitoring. It has been demonstrated that salsa and guacamole are frequent vehicles of foodborne disease outbreaks in the United States.[190] Unsurprisingly, fresh serrano and jalapeno peppers used in these food items have caused huge multistate *Salmonella* outbreaks.[191,192] Fresh salsa and guacamole require careful preparation and storage, and food prevention strategies based upon the HACCP principles can greatly help to reduce the incidence of foodborne disease, as well as to maintain monitoring of these food items.

Focus is often targeted on the inputs to food, water, or agricultural production, and when a product leaves the plant, that attention may be dis-

continued. The time and route of delivery, as well as the security of the transportation, may be the most vulnerable points in the continuum and should not be overlooked when planning security. Studying incidents of nonpurposeful foodborne pathogen contamination, such as the 1985 Minnesota salmonellosis outbreak,[193] may reveal potential avenues for purposeful outbreak scenarios. This outbreak and many others demonstrate that foodborne bioterrorism might have greater chances of success when pathogens are introduced after processing and as close to consumption as possible, thus circumventing opportunities for dilution and destruction by cooking or pasteurization.

Implementing rational employee hiring and accountability procedures may also effectively mitigate food, water, or agricultural vulnerabilities.[186] Additional strategies include implementing procedures for laboratory testing and monitoring, reporting and investigating inspection discrepancies, and ensuring computer and information security.[186]

Various disease surveillance systems (covered in greater details in other chapters) are in place, including local, state health agency, and CDC programs to track and identify trends in foodborne illness, including FoodNet,[194] PulseNet,[195] CalciNet,[196] WBDOSS,[197,198] and syndromic surveillance systems such as RODS[199] and BioSense.[200] Additional methods to inspect and protect food and water supply chains, and rapidly integrate disease surveillance, are being actively examined and implemented.[201]

Furthermore, under the Food Safety and Modernization Act of 2010 the Food and Drug Administration has proposed a rule that would require the largest food businesses in the United States and overseas to take measures to prevent food facilities from being targeted by intentional attempts to contaminate the food supply.[202] Under the proposed rule, food facilities would be required to have a written food defense plan addressing significant vulnerabilities in their food production process, and to take measures to address these vulnerabilities, establish monitoring measures and corrective actions, confirm that the system is working, and ensure that workers assigned to vulnerable areas receive suitable training and maintain records.[202]

SUMMARY

Any biological pathogen, whether bacteria, virus, toxin, or parasite, has the potential to be used in a terrorism context. Historical examination of both purposeful and inadvertent food and waterborne disease outbreaks can greatly assist in understanding how such events occur and how they may be prevented. A comprehensive understanding of animal production and crop farming, as well as food production and distribution, is required to ensure protection for the agricultural industry from terrorism events. Absolute safety of the food supply is perhaps an unattainable goal, but should be the benchmark for which all food protection and agricultural efforts are directed.

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Chapter 4

CONSEQUENCE MANAGEMENT: THE LOCAL AND NATIONAL RESPONSE

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INTRODUCTION

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INTRODUCTION

Consequence management is critical to minimizing the long-term impact from any natural or manmade disaster. A common aim of organizations that seek to induce terror is to cause maximum disruption to societies with no regard for the impact on human life. Effective consequence management will minimize a disaster's impact on a society, its people, its infrastructure, and its economy. It will deliver the right solutions at the right time to the right locations in a coordinated and controlled response. Many, but not all, consequences of a catastrophic event are predictable, and many catastrophic events share common types of consequences. An effective consequence management plan focuses on the critical functions and capabilities necessary to span multiple types of disasters that effectively minimize the disaster's impact. The plan must be comprehensive, flexible, and scalable. It must be adaptable to variable outcomes of varying magnitude and potential cascading effects of catastrophic events that may require a rapid transition from the initial plan to a more comprehensive one.

Constructing an effective consequence management plan starts with understanding the threats to a populace, geographic location, or specific entity and the potential impact of those threats on all facets of the affected area. It must identify local response capabilities and functions and develop an activation strategy for timely implementation. It should also demonstrate an understanding of regional, state, and federal response capabilities and functions and outline a mechanism for requesting assistance. Effective consequence management starts at the local level, but can rapidly escalate with the need to coordinate higher-level supportive response with ongoing local response and recovery efforts.

Federal response and recovery key planning considerations and responsibilities are identified in the National Response Framework (NRF) and the National Disaster Recovery Framework (NDRF), available for download from the Department of Homeland Security (DHS) and the Federal Emergency Management Agency (FEMA) digital libraries. They identify essential support functions and recovery support functions that guide local, state, and interagency federal all-hazards planning for response and recovery. The Biological Incident Annex to the NRF outlines the actions, roles, and responsibilities associated with a human disease outbreak of known or unknown origin requiring federal assistance. These documents emphasize a common theme: response and recovery will start at the local level and local involvement throughout is critical to success. Robust local planning to reduce an entity's vulnerability to the threats of biological incidents, with a well-thought plan for disaster response and recovery that meshes well with state and federal assistance plans, can save lives, minimize impact, and defeat terrorist objectives.

CONSEQUENCE MANAGEMENT

The definition of consequence management has evolved over time. At one time a clear separation existed between crisis management and consequence management. In a November 20, 2003 hearing before the House of Representatives, Subcommittee on Crime, Terrorism, and Homeland Security, Mr. Howard Coble defined crisis management as actions taken to anticipate, prevent, or resolve a threat, with consequence management fulfilling the cleanup and restoration functions after an attack.[1] To truly mitigate the short-term and long-term impacts of catastrophic events, one must think and plan over a continuum of prevention, protection, and mitigation through response and recovery.

The NDRF emphasizes that community recovery can be accelerated through a community's efforts in predisaster preparedness, mitigation, and recovery capacity building.[2] Recovery efforts must not interfere with immediate response efforts to preserve life and health and maintain critical infrastructure. However, immediate response efforts can mitigate long-term effects and the overall impact of the disaster with significant impact on postdisaster recovery—positively or negatively—and the two should be planned and executed in harmony.

The NDRF emphasizes that recovery encompasses far more than the restoration of a community's physical structures; it must also provide a continuum of care to meet the needs of the affected community members who have experienced financial, emotional, or physical impacts.[2] Good communication and coordination early and throughout a disaster response will help inform long-term recovery planning as well as prevent selection of short-term solutions that may result in negative long-term impact. Integration among the frameworks is considered one of the key themes of the National Preparedness Goal (NPG).[3]

Planning must be risk based and risk assessments must be comprehensive and standardized. Planners, regardless of their affiliation, must understand what threats have the greatest potential impact on their area of responsibility. Threat and hazard identification and

risk assessment guidance is provided in the Comprehensive Preparedness Guide 201.[4] The guide describes a five-step process for threat and hazard identification and risk assessment:

1. identifying threats and hazards of concern;
2. giving them context;
3. examining core capabilities;
4. establishing capability targets; and
5. applying results.

The Army Techniques Publication No. 5-19 outlines a risk management approach to analyzing and mitigating hazardous operations. Core components of risk management are conducting a hazard analysis based on source, mechanism, and outcome of each hazard; an assessment of risk based on the probability the hazard will be experienced; and the severity of the outcome, followed by development of a strategy to mitigate risk that focuses on lowering the probability or lessening the severity resulting in an acceptable level of residual risk. Risk assessment must be continuous and updated throughout the course of a response to hazardous conditions, and it must be specific to the environment, local infrastructure, and population. Severity of outcomes will likely vary significantly between heavily populated areas and sparsely populated areas and could vary considerably within the same city depending on what part of the city is affected, time of day, or whether the incident occurs during a workweek, weekend, or special event.

Both risk management systems stress the importance of identifying hazards through lessons learned from past events and subject matter expert opinion, understanding how they may affect an entity's operations, identifying capabilities that can mitigate the impact of the hazard, and proper resourcing to implement the necessary control measures. These systems can assist planners with selecting controls to mitigate outcomes, inform predisaster resourcing to enhance preparedness (eg, establishment of memoranda of agreement), decrease the residual risk, and assist with comprehensive consequence management planning. An example of where this can assist consequence management following a population's exposure to a biological agent or toxin is to factor in the mechanism by which the resultant disease can spread.

Some aspects of consequence management following exposure to a biological agent that causes contagious disease could significantly differ from one that is not. An overreaction for a disease-causing agent that is not contagious could cause unnecessary negative impact to the local infrastructure and economy and further complicate long-term recovery. Conversely, a lack of planning or inability to control the spread of a contagious disease could result in uncontrolled spread beyond the contamination zone with severe to catastrophic impact on life, health, infrastructure, and economy over a broad geographic region. A contagion will likely require implementation of quarantine and/or isolation as a control measure, but quarantine and/or isolation may not be appropriate for an agent that does not cause a contagious disease. The mechanism by which a hazard produces negative outcomes is important. When local planners do not have subject matter experts available to assist with planning, they should seek assistance from county, state, and federal public health professionals.

The general population does not understand the unique differences among the various potential pathogens, their mechanisms of transmission, and the differences in risk created by each. Misinformation disseminated through rumors or poorly informed news outlets can create additional challenges to the response and recovery effort. It can lead to confusion as well as a loss of confidence in those leading the response and recovery effort. Timely accurate information dissemination to the community is important, whenever a threat to public safety occurs.

An analysis of the human response to the catastrophic events of September 11, 2001 reveals that fear during a crisis situation does not automatically result in panic, and the negative impact of fear may manifest more significantly during the consequence management period when considerable uncertainty exists.[5] It revealed a tremendous spirit of cooperation and compassion among the affected population during the crisis period. Timely release of accurate information can mitigate the effects of misinformation and facilitate a spirit of teamwork throughout response and recovery. Close coordination of press releases with public affairs professionals is critical to accurate information dissemination.

One intending to induce terror will rely on a hazard's natural and intended consequences but will also benefit from unintended consequences that are a product of poor or narrowly focused planning, that is not flexible and scalable, resulting in an inefficient response with poor communication. Similarly, the magnitude of impact from a naturally occurring outbreak of infectious disease can be minimized or magnified by the quality of response planning and efficiency of execution.

Selecting the appropriate medical countermeasures and understanding the potential effects will rely on accurate agent identification at the time of the incident, but detailed planning for specified agents would be an inefficient approach to general consequence manage-

ment and would risk not having an actionable plan in place when needed. Local, state, and federal response and recovery planning can leverage common mechanisms and common potential outcomes to be prepared for many potential threats rather than constructing plans for every possible disease causing agent.

CONSEQUENCES OF A BIOLOGICAL INCIDENT

Biological incidents may be one of the most challenging threat conditions for planning consequence management. Response and recovery for most disasters follow a major catastrophic event that is relatively rapidly definable in scope and magnitude of impact. Conversely, it is unlikely that a biological incident will present as a well defined major catastrophic event. However, it carries the potential for significant casualties and major disruption to a community, region, or nation's infrastructure and economy, with a significant fear factor and potential for panic among a nation's population. If it involves a contagion, it also has the potential to spread well beyond the original target area in a short period of time. If it involves a zoonotic disease-causing agent, or is equally infectious to a local animal population, secondary spread from human–animal contact may persist and human health efforts must be synchronized with that of the animal health and feral animal control industries. With the exception of an emerging infectious disease with pandemic potential, where initial cases have already been identified, no warning or opportunity will likely occur for leaders to proactively surge or position resources. A scalable response must be part of a community's and nation's normal framework.

A series of framework documents can guide planners through consequence management planning and execution:

- National Prevention Framework,
- National Mitigation Framework,
- NRF, and
- NDRF.

All disasters have the potential for cascading effects, especially for a biological incident. Those intending to induce terror will attempt to leverage this potential to achieve their objectives and the response and recovery efforts must control them to minimize the impact on a society. Whether a biological incident is intentional, accidental, or results from a natural disease outbreak, it is important to rapidly identify the potentially affected area and population and control movement. Without these controls rapidly implemented, the zone of potential contamination will grow. Concurrent with controlling movement into and out of the affected area, the agent or agents involved must be rapidly identified. Differing biological agents have differing incubation periods, environmental survivability, and modes of transmission. Appropriate medical countermeasure and decontamination procedure identification and implementation timelines will rely on accurate agent identification. If animals and insect vectors can be potential sources of residual infection and sources for spreading the disease causing agents, the appropriate subject matter experts must be included in response and recovery planning.

Sick people with relatively generic conditions show up in emergency departments, urgent care clinics, and primary care clinics on a daily basis. It is likely that a disaster will not be declared until their condition is identified as part of a natural disease with pandemic potential or tied to an act of bioterrorism. By this time the consequences of the causative event are being experienced, and the challenge of determining where it started and what caused it exists. The immediate consequences are obvious and rely on availability of the appropriate diagnostic assays and medical countermeasures. Some critical questions must be immediately answered:

- Is this disease typical of the human population or geographic location?
- Is it contagious?
- Are animals also affected?
- Is it zoonotic?
- Does the pattern of disease in the population suggest a natural outbreak or an intentional release?

Answers to these questions will inform immediate response and identify potential cascading effects and consequences that must be managed. If the incident is determined to be suspicious for an intentional release, a criminal investigation must immediately accompany the public health and epidemiologic investigations. These efforts must be synchronized to ensure no disruption to response efforts intended to preserve life and health while also preserving evidence to facilitate the criminal investigation.

A biological incident will present a significant amount of uncertainty for professionals responding to the incident and this will be magnified throughout the community. Timely accurate dissemination of information can mitigate a public reaction that may compound the problem if not appropriately managed.

The National Incident Management System, which calls for a unified approach to multiagency coordination during response and recovery operations,

emphasizes the unified approach concept based on chain of command, unity of command, unity of effort, and when implemented, unified command.[6] Unity of effort is critical to disseminating consistent informative messages at the right time to assist with managing public fear and reaction. Conflicting information from multiple agencies creates confusion, a lack of confidence in the response and recovery effort, and potential panic.

The anthrax (*Bacillus anthracis*) attacks of 2001 provide lessons on the value of effective risk communications and public reaction. Confusion following the initial reports was widespread on a national scale. Examples of communication failures include the following:

- 46% of the population thought anthrax was a contagious disease.
- 70% of the New Jersey population were concerned that they or someone close to them had been exposed.
- Agencies were overwhelmed by requests for information.
- Reports of discontent with both quality and timeliness of information were heard.[7]

An example of risk communication success came from a multiagency task force in New Jersey that was reported as beneficial for enhancing cooperation between law enforcement and health organizations and reducing tensions. Law enforcement and health organizations approach communications differently, with law enforcement leaning toward secrecy and public health valuing openness.[7] By managing disparate philosophies through a task force or unified command element, messaging can be less confusing for the recipients, with greater efficiency in providing informative messages in a timely manner that do not compromise criminal investigations and serve to palliate public fear and reaction.

Preserving the life and health of an affected population must be the primary concern during response and recovery, but consequence management planning and execution for a biological incident cannot be narrowly focused on individual patient care. If a biological agent is intentionally dispersed, the dispersion method must be rapidly characterized to determine the extent of contamination and identify the affected human and/or animal population(s). If agent dispersion is covert and the first indication of an act of bioterrorism or criminal act involving a biological agent is one or more patients presenting with clinical disease, determining the extent of contamination and the affected population will be challenging but critical to effectively managing the consequences. Once the affected area is determined, access to and egress from the area must be controlled, but controls must be tailored to the type of agent(s) identified.

A detailed assessment of the affected area that includes personnel and equipment movement, wild and domestic animal populations and movement, and surface water flow patterns must be conducted to determine the potential for contamination spread and other potentially affected populations. If the affected area involves significant community services, planners will need to determine how to continue providing those services to the unaffected community to minimize the incident's overall impact, especially if it involved any services critical to the response and recovery effort. The initial assessment of causative agent, method of dispersion, and extent of contamination will also assist health providers and planners on selection of medical countermeasures and the potential number of expected human casualties. If the extent of contamination is large or multifocal, or if the causative agent is a contagion, patient care facilities and medical countermeasures could be rapidly overwhelmed, necessitating the need for rapid coordination of external support. Plans and support agreements to manage this potential consequence must be in place before an incident. These agreements are an area where neighboring communities and neighboring states can seek mutual aid and support when one or the other is affected by a catastrophic event.

The impact of a biological incident will likely extend well beyond the primary concern of human health considerations. Whether secondary hazards from a biological incident are real or perceived, they will have significant impact on the affected entity and surrounding population. Biological incidents have the potential to overwhelm local healthcare resources and render any business or public service provider inoperable. If a community's healthcare facility and emergency response capabilities are contaminated, it is imperative that a backup plan for care and emergency response is in place. How effectively a community conducts waste management will either create or mitigate secondary concerns and the impact of accumulating waste. Waste may also require special treatment that is not part of a community's standard operating procedures, and special assistance may be required.

Communities should develop continuity of operations plans to identify backup resourcing for critical services. Businesses should also develop continuity of operations plans to protect their livelihood in case they are directly or indirectly affected by an emergent situation and unable to occupy their normal place of business. The former $3.8 million American Media Inc. building in Boca Raton, Florida, was quarantined on October 10, 2001, sold for $40,000 in April 2003, and

was not reported to be clear of *Bacillus anthracis* until February 7, 2007.[8] The Hart Senate Office Building reopened after it cost $27 million to decontaminate it. Economic impact on an affected business can be catastrophic and a protracted disruption to services can occur when a public service facility is affected.

LOCAL AND NATIONAL RESPONSE

The NPG suggests that successful consequence management of a biological incident will result from capabilities being available across the whole community to prevent, protect against, mitigate, respond to, and recover from the threats and hazards that pose the greatest risk.[9] The NPG and supporting framework documents emphasize that an effective response starts at the local level with individuals, community organizations, the private and nonprofit sector, faith-based organizations, and local governments all having a critical role with support from the state and federal governments.

The NPG identifies core capabilities for five mission areas: (1) prevention, (2) protection, (3) mitigation, (4) response, and (5) recovery. It recognizes that the core capabilities listed are ambitious and will require a national effort involving the whole community to be effective, with three core capabilities spanning all five mission areas: (1) planning, (2) public information and warning, and (3) operational coordination.[9] The NPG lists five key findings from the Strategic National Risk Assessment; two key risk areas correlate with the subject of this chapter: a virulent strain of pandemic influenza and terrorist use of weapons of mass destruction.[9]

Planners at all levels should assess their level of readiness to manage consequences associated with these risks, but not limit their planning to just these risks. Every community and state may have differing priority lists for planning. Military planners routinely assess two types of risks to operations: most likely and most dangerous. This approach can also prove valuable for planners as they develop local and state core capabilities for effectively managing risks posed by various biological threats.

Although planners should be familiar with several framework documents, the remainder of this chapter will primarily focus on guidance from the NRF, the NRF Biological Incident Annex, the NDRF, and other supportive documents. Framework documents emphasize the significance of local readiness and response and provide federal agency level guidance that can be tailored to all levels of government planning. With a few exceptions, federal assets will not be mobilized until local and state capabilities have been or likely will be exceeded and a state's governor requests federal assistance under the Stafford Act. These documents provide valuable guidance for planning at all levels.

The NRF lists 14 core capabilities, 15 emergency support functions, and four priorities for the response mission area that local and state planners can use as a template for their planning activities. Core capabilities include the following:

1. planning;
2. public information and warning;
3. operational coordination;
4. critical transportation;
5. environmental response/health and safety;
6. fatality management services;
7. infrastructure systems;
8. mass care services;
9. mass search and rescue operations;
10. on-scene security and protection;
11. operational communications;
12. public and private services and resources;
13. public health and medical services; and
14. situational assessment.[10]

Emergency support functions (ESF) include the following:

1. ESF1 transportation;
2. ESF2 communications;
3. ESF3 public works and engineering;
4. ESF4 firefighting;
5. ESF5 information and planning;
6. ESF6 mass care, emergency assistance, temporary housing and human services;
7. ESF7 logistics;
8. ESF8 public health and medical services;
9. ESF9 search and rescue;
10. ESF10 oil and hazardous materials response;
11. ESF11 agriculture and natural resources;
12. ESF12 energy;
13. ESF13 public safety and security;
14. ESF14 (replaced by the NDRF); and
15. ESF15 external affairs.

Response mission priorities include the following:

1. save lives;
2. protect property and the environment;
3. stabilize the incident; and
4. provide for basic human needs.[10]

Coordinating agencies are identified for each of the emergency support functions at the federal level; local and state planners should identify assets for planning at their respective levels. Objectives for each of the core capabilities listed in the NRF are summarized in Table 4-1. Local planners will likely not have resources available to meet all of these objectives, but the list can assist them with determining what local assets need to be factored into a local response plan and what support requirements are needed to coordinate with neighboring communities or the private sector through support agreements, or requests from county, state, or federal partners.

The effective response to a catastrophic event will require meeting many of the core capability objectives through local assets, at least for initial response and later to augment state or federal response efforts. Local assets may include individuals, the private sector, nongovernmental organizations, and neighboring communities.

TABLE 4-1

CORE CAPABILITIES AND OBJECTIVES

Capability	Objectives
Planning	Conduct a systematic process engaging the whole community in the development of executable strategic, operational, and/or community-based approaches to meet defined objectives.
Public Information and Warning	Deliver coordinated, prompt, reliable, and actionable information to the whole community through the use of clear, consistent, accessible, and culturally and linguistically appropriate methods to effectively relay information regarding any threat or hazard and the actions being taken and the assistance being made available.
Operational Coordination	Establish and maintain a unified and coordinated operational structure and process that appropriately integrates all critical stakeholders and supports the execution of core capabilities.
Critical Transportation	Provide transportation (including infrastructure access and accessible transportation services) for response priority objectives, including the evacuation of people and animals, and the delivery of vital response personnel, equipment, and services to the affected areas.
Environmental Response/Health and Safety	Ensure the availability of guidance and resources to address all hazards, including hazardous materials, acts of terrorism, and natural disasters, in support of the responder operations and the affected communities.
Fatality Management Services	Provide fatality management services, including body recovery and victim identification to provide temporary mortuary solutions, sharing information with Mass Care Services for the purpose of reunifying family members and caregivers with missing persons/remains, and providing counseling to the bereaved.
Infrastructure Systems	Stabilize critical infrastructure functions, minimize health and safety threats, and efficiently restore and revitalize systems and services to support a viable resilient community.
Mass Care Services	Provide life-sustaining services to the affected population with a focus on hydration, feeding, and sheltering to those with the most need, as well as support for reunifying families.
Mass Search and Rescue Operations	Deliver traditional and atypical search and rescue capabilities, including personnel services, animals, and assets to survivors in need, with the goal of saving the greatest number of endangered lives in the shortest time possible.
On-scene Security and Protection	Ensure a safe and secure environment through law enforcement and related security and protection operations for people and communities located within affected areas and for all traditional and atypical response personnel engaged in lifesaving and life-sustaining operations.
Operational Communications	Ensure the capacity for timely communications in support of security, situational awareness, and operations by any and all means available between affected communities in the impact area and all response forces.
Public and Private Services and Resources	Provide essential public and private services and resources to the affected population and surrounding communities to include emergency power to critical facilities, fuel support for emergency responders, and access to community staples (eg, grocery stores, pharmacies, and banks) and fire and other first response services.
Public Health and Medical Services	Provide lifesaving medical treatment via emergency medical services and related operations, and avoid additional disease and injury by providing targeted public health and medical support and products to all people in need within the affected area.
Situational Assessment	Provide all decision makers with decision-relevant information regarding the nature and extent of the hazard, any cascading effects, and the status of the response.

Data source: Department of Homeland Security. *National Response Framework.* 2nd ed. Washington, DC: DHS; May 2013.

Individuals within a community possess talents and experience that can be organized for response through community organizations. Individuals should participate in community preparedness planning activities and develop household emergency plans.[10] Individuals can also participate in FEMA's Community Emergency Response Team Program, which educates people on how to prepare for hazards that may affect their area and trains them in basic disaster response skills, such as fire safety, light search and rescue, team organization, and disaster medical operations.[11] Community Emergency Response Team Program trained individuals can play a critical role in assisting with local planning and response. Private sector entities can support local emergency management and should also participate in community preparedness planning activities.[10] Private sector entities should also conduct continuity of operations planning within their own organization to establish a plan that will foster their continued support or service to the community while also preserving their livelihood.

Nongovernmental organizations may factor into any level: local, state, or federal response. They manage volunteers and resources to support incident response through collaboration with responders, all levels of government, and other agencies and organizations.[10]

Neighboring communities can play a critical role in consequence management for a biological incident. Community dynamics can be significantly disrupted within the contamination zone of a biological incident, but life outside the contamination zone will continue with no change in requirement for services and support. If a community's critical services are located within the contamination zone and are rendered inoperable because of real or perceived contamination, then mutual aid agreements among communities can fill critical gaps in the response effort as well as continuation of services to the unaffected parts of the community.

When local resources are exhausted or prove to be inadequate, local authorities may seek county or state assistance; in some situations, local authorities may seek assistance directly from the federal government for non-Stafford Act incidents.[10] Some federal departments or agencies, using funding sources other than the President's Disaster Relief Fund, can conduct or lead federal response actions under their own authorities.[10] Examples include immediate lifesaving assistance, wild-land firefighting, response to an agricultural disease, cybersecurity incidents, and oil and hazardous substance response operations.[10] The Secretary of the Department of Health and Human Services (DHHS) has the authority to take actions to protect the public health and welfare and declare a public health emergency.[10]

State governors are responsible for the public safety and welfare of their state's residents. Their responsibilities and authorities include making, amending, or suspending orders or regulations associated with response; communicating to the public; coordinating with tribal governments; commanding the state military force (National Guard personnel not in federal service); coordinating assistance from other states; and requesting federal assistance.[10] A state's response to emergency situations is coordinated through the state emergency management agency.

Numerous state departments and agencies have a role in response and recovery, but the National Guard is one of the governor's key assets for a biological incident. National Guard members can be valuable to consequence management because of their expertise in emergency medical response; communications; logistics; search and rescue; civil engineering; and chemical, biological, radiological, and nuclear response, planning, and decontamination.[10] Weapons of mass destruction/civil support teams are highly specialized National Guard units designed to provide unique capabilities for response to chemical, biological, radiological, or nuclear incidents, primarily in a Title 32 operational status within Washington, DC, the United States, its territories, and its possessions.[12]

Federal financial aid or other support to response, recovery, and mitigation efforts are authorized following a Stafford Act emergency or major disaster declaration by the president. An emergency declaration is more limited in scope, provides fewer federal programs, and is not normally associated with recovery programs, but it may be used before an incident to mitigate the threat of a potential catastrophe.[10] Most of the president's authority under the Stafford Act has been delegated to the FEMA administrator through the Secretary of Homeland Security.[10] A state's governor may request federal assistance through the FEMA regional administrator when the situation is considered beyond the capabilities of the state and affected local government.[10]

The DHHS is the coordinating agency for the biological incident annex to the NRF. Federal government objectives for response to a biological incident—naturally occurring or as an act of terrorism—are as follows:

- detect the event through disease surveillance and environmental monitoring;
- identify and protect the population(s) at risk;
- determine the source of the disease;
- assess the public health, law enforcement, and international implications;
- control and contain any possible epidemic;
- augment and surge public health and medical services;

- identify the cause and prevent the recurrence of any potential resurgence, additional outbreaks, or further spread of disease; and
- assess the extent of residual biological contamination and conduct response, restoration, and recovery actions.[13]

Detection of a biological incident may be by the presentation of disease in humans or animals or environmental surveillance systems, or by acts of bioterrorism detected though the normal operations of other cooperating departments and agencies.[13] The National Biosurveillance Integration System is a tool that supports detecting disease outbreaks by leveraging data from multiple surveillance systems that monitor human health, animal health, plant health, and food and water.[13] Monitoring for dangerous pathogens in some heavily populated places is accomplished through the BioWatch program, which serves as an early detection and warning system.[14] This program has been criticized for creating false alarms, but those criticisms probably come from individuals who do not fully understand the technologies used, intent of the program, and confirmatory process that follows an alert. DHS partners with public health laboratories through the Laboratory Response Network to rapidly confirm any alerts from BioWatch systems.

Detection technologies need to be rapid and sensitive; they need to ensure that no false negatives occur. Specificity is ensured through the laboratory confirmatory process that follows. People will live with a few false alarms, but they will become sick and potentially die from false negative results at the detection level. It is unlikely that samples will be forwarded for confirmatory analysis if results are negative at the detection level, so these systems should be judged more on their potential to prevent false negatives than false positives. These systems are value added if they are not over interpreted before confirmatory analysis. Claims of false positives have been characterized as unsubstantiated, with more than 7 million tests performed by public health laboratories and no false positives.[14]

DHHS convenes a meeting of ESF #8 partners, after notification of a credible threat or disease outbreak, to assess the situation and determine appropriate public health and medical actions.[13] If the threat or disease outbreak is suspected to be tied to a criminal or terrorist act, the Federal Bureau of Investigation will lead a concurrent criminal investigation and possibly establish a joint operations center.[13] Joint operations centers are valuable to establishing unity of effort. Agencies with disparate primary objectives will be working simultaneously toward a common outcome, but do not always fully understand each other's mission priorities; synchronization of efforts is critical to mission success.

It is important for first responders to understand that they may be working within a crime scene and that all materials may have evidentiary value, but this fact cannot compromise mitigating the immediate threats to life and health. If a criminal or terrorist incident initially presents as disease in humans or animals, criminal intent may not be apparent for some time and evidence may already be compromised. The Laboratory Response Network is used to test samples whenever a credible threat of a biological crime or act of terrorism exists.[13] If contamination of food is suspected the Food Emergency Response Network, a complementary system to the Laboratory Response Network, may be used for food sample analysis.[13]

Other federal agencies will support DHHS during a biological incident response. The DHS will serve as the incident coordinator. The Environmental Protection Agency will develop and implement sampling strategies when a potential for environmental contamination exists. The Department of Agriculture will provide support for an outbreak of an agriculturally significant zoonotic disease or human foodborne pathogen. Federal public announcements, statements, or press releases will be coordinated with the DHS Office of Public Affairs, consistent with ESF #15.[13]

An epidemic resulting from the introduction of a contagious biological agent into a population is one of the most significant—and likely the most dangerous—potential consequences of a biological incident. Effectively managing this potential consequence relies on the following:

- rapid detection, and identification and confirmation of the biological agent;
- identification of the population at risk;
- determination of how the agent is transmitted;
- determination of appropriate medical countermeasures;
- administration of countermeasures;
- rapid dissemination of safety information to the public; and
- control and containment strategies.

Planning must include worst-case scenario branches for mass casualties if early control measures are not effective and containment is not achieved, requiring augmentation and surging of health and medical resources in order to track and prevent additional disease outbreaks.

DHHS assists partner public health and medical authorities with epidemic surveillance and coordination, and it will assess the need for increased surveillance. DHS, with partner organizations, coordinates timely,

consistent, accurate, and actionable information dissemination. The public health system, starting at the local level, initiates appropriate protective measures for the affected population, including all workers involved in incident response. DHHS, with partner organizations involved, evaluates the need for isolation, quarantine, or shelter-in-place measures to prevent spread of disease. If isolation and/or social distancing are recommended, the affected state's governor may implement these measures under state or local legal authorities. Tribal leaders also possess this authority under tribal legal authority.

DHHS may take appropriate federal actions to prevent the import or interstate spread of disease. If the source of the disease outbreak is identified as originating outside the United States, DHHS works with DHS and other agencies to identify and isolate persons, cargo, mail, or conveyances that may be contaminated. If it is determined that food, animals, and other agricultural products need to be quarantined, livestock or poultry need to be vaccinated or depopulated, and/or movement of animals and equipment need to be restricted, DHHS will work with the Department of Agriculture and other partner organizations. DHHS works through the Department of State to notify affected foreign governments if foreign nationals are subjected to isolation and/or quarantine.[13]

The ability to care for sick and/or potentially exposed people is one of the most critical response requirements that must be incorporated into predisaster response planning. The Strategic National Stockpile is a national repository of medical countermeasures, vaccines, and medical supplies stored in strategic locations.[15] Division of Strategic National Stockpile personnel, from the Centers for Disease Control and Prevention's Office of Public Health Preparedness and Response, will assist as local and state health departments prepare for receipt, distribution, and dispensing of medical countermeasures from the Strategic National Stockpile.[15] These medical countermeasures, vaccines, and medical supplies are free to the public, and states have plans to receive and distribute them once federal and local authorities agree that they are needed.[16] Strategic National Stockpile supplies include 12-hour push packages, CHEMPACKs (program that provides antidotes to nerve agents [three countermeasures used concomitantly] for prepositioning by state, local, and tribal officials), and federal medical stations. The 12-hour push packages contain 50 tons of a broad spectrum of medical assets and can be delivered to any state in the continental United States within 12 hours from the decision to deploy; if the incident requires additional or different supplies, they can be delivered within 24 to 36 hours.[15] Federal medical stations are rapidly deployable and modular, stocked with beds and supplies to care for up to 250 patients for up to 3 days.[15]

As with other aspects of the integrated local, state, and federal response effort, local technical expertise and local planning will be critical to efficient and successful delivery of medical care to those who need it. Centers for Disease Control and Prevention developed the public health preparedness capabilities/national standards for state and local planning that can assist state and local public health officials with planning for this and other critical public health planning considerations. It identifies 15 public health preparedness capabilities under six domains[17]:

1. biosurveillance;
2. community resilience;
3. countermeasures and mitigation;
4. incident management;
5. information management; and
6. surge management.

This downloadable document is an excellent planning guide that links planning and execution activities back to NRF emergency support functions and provides links to additional resources.

Beyond the challenge of medical countermeasure availability and distribution, a biological incident may challenge the ability of healthcare systems to adequately care for large numbers of patients that exceed local capabilities and capacities, and it will likely affect their ability to continue providing a standard of care to the local community for routine health issues. Maintaining medical system resiliency may require regional, state, or federal coordination and medical surge capacity and capability. The medical surge capacity and capability management system was developed to provide a systems-based approach for managing the complexity of mass casualty or complex incidents.[18] Surge capacity is the ability to respond to a markedly increased number of patients. Surge capability is the ability to address unusual or very specialized medical needs. The medical surge capacity and capability management system is consistent with the National Incident Management System and guides public health and medical response through a six-tier approach, escalating from management of individual healthcare assets to federal support to state, tribal, and jurisdictional management.

RECOVERY

Recovery operations focus on returning the affected region or entity, as closely as possible, to predisaster conditions. Initiation of recovery efforts does not require full completion of response operations, but the transition process must be well synchronized with any continuing response efforts. Many components of the response effort will also influence recovery activities.

As healthcare transitions from emergent and temporary medical care, activities must ensure continuity of care and reestablishment of any disrupted healthcare capabilities. Continuity of care may need to be established through temporary facilities until services are fully restored; candidate facilities should be identified during consequence management planning activities. Surveillance should be initiated during response, as discussed in the previous section, and continued through recovery until health officials determine that the discovery of new cases has met criteria for discontinuation.

Effective messaging by public health professionals can serve to mitigate public fear and prevent panic. Several people will be identified, throughout response and recovery, who may benefit from counseling and behavioral health services. These services should be restored or made available as soon as possible. Lessons learned from previous disasters suggest that during the transition and recovery period, public fear can increase. During this period people will have gained awareness of the morbidity and mortality associated with the infectious agent or toxin and will have continued—possibly escalating—fear about exposure to this invisible threat.

Ineffective messaging may have contributed to public fear and panic during the 2001 Amerithrax incident. Many people thought anthrax was a contagious disease, and because the infectious agent was delivered in a powdered form there was widespread fear of powders in general. People were more aware of powders and powder-appearing residues after the incident. Powders associated with many normal activities that went unnoticed or created no concern before the incident suddenly created concern, fear, and panic. The US Army Medical Research Institute of Infectious Diseases and many other laboratories involved in the recovery effort received thousands of samples for analysis that normally have an innocuous powder associated with them. Effective accurate communications may have mitigated some of these concerns and will remain important throughout any recovery.

Exposed populations and contaminated buildings, equipment, and environments will likely be identified during the response effort, but continued surveillance will remain important to identify additional cases of human or animal disease and potential contamination spread that will need to be included in decontamination efforts. Decontamination can and will likely be challenging. Its effectiveness will depend on accurate identification of the contaminating infectious agent or toxin; assessment of primary and secondary areas of contamination; and selection of suitable decontamination reagents, equipment, and methods that factor in effectiveness for the contaminating agent and the environment. Appropriate subject matter experts should be included in planning and executing decontamination.

Personnel involved in recovery operations will not have the benefit of established clearance strategies for reoccupation of contaminated facilities or resumed use of contaminated equipment for all potential biological agents. Members of the Environmental Protection Agency and the Centers for Disease Control and Prevention published an interim clearance strategy for a building or an outdoor environment after an incident involving *Bacillus anthracis* in July 2012. It was determined that no detection of viable spores is the best practicable clearance goal,[19] which is a sound goal for *B anthracis* as well as many other potential biological agents. It infers that the agent identification technology used will identify viability as well as continued presence of the pathogen on or in the sampled item.

Some of the more sensitive agent identification technologies (nucleic acid amplification and antigen detection) will not demonstrate agent viability. Agents killed or neutralized during decontamination may still be detected by these technologies and not properly inform clearance decisions. Cleanup procedures could be unnecessarily prolonged with no added benefit if decisions are being made based on technologies that do not aid the risk assessment procedure by demonstrating agent viability. Local or state public health officials or property owners will likely make the final decision on clearance.[19] However, the lack of established standards and complexity of this decision process will likely necessitate the support of external subject matter experts.

Establishing transportation routes becomes critical during both response and recovery to facilitate response and recovery mitigation activities as well as continue providing critical services and support inside the contaminated area. Once a biological incident has occurred, containment becomes important

to all aspects of the management strategy. Factoring containment considerations into all subsequent planning will prevent the incident from growing in scale and magnitude, minimizing impact on human health, infrastructure, and economy. Strategies will vary depending on the situation and conditions, but some basic principles can be applied to all. Whenever one is dealing with biological contamination, it is beneficial to establish at least three zones:

1. known or high probability to be contaminated (hot zone);
2. not expected or low probability to be contaminated (warm zone); and
3. expected to be clean (cold zone).

Operational procedures should be established for each zone that facilitate the movement of necessary supplies, personnel, and equipment to sustain operations and facilitate recovery without spreading contamination. If a clean corridor cannot be established through the warm zone to the hot zone, handoff procedures will need to be established for cross-zone movement. Decontamination procedures at each handoff point will need to be established for any movement from hot zone to warm zone and from the warm zone to the cold zone. One strategy may be to have dedicated equipment in each zone that will facilitate the movement of personnel and supplies from the cold zone to the hot zone and sustain operations in the warm zone and hot zone. Personal protective equipment requirements must also be established for each zone to prevent secondary contamination to workers.

The NDRF promotes nine core principles for recovery success:

1. individual and family empowerment;
2. leadership and local primacy;
3. predisaster recovery planning;
4. partnerships and inclusiveness;
5. public information;
6. unity of effort;
7. timeliness and flexibility;
8. resilience and sustainability; and
9. psychological and emotional recovery.[2]

It promotes a concept of all-community involvement in recovery efforts to ensure that no groups of people and their unique interests are excluded during the recovery effort and that services are made equally available to everyone as all affected members of a community attempt to rebound from their losses. It affirms that local leaders and local governments maintain a primary role even when their response capabilities have been overwhelmed and state or federal assistance is required. It recognizes that partnerships and collaborations with unity of effort are essential to successful recovery and emphasizes that compliance with the principles of equal opportunity and civil rights must be upheld. It further emphasizes the importance of clear, consistent, culturally appropriate, and frequent communications to the affected public. Timeliness and flexibility are emphasized to minimize missed opportunities and foster the ability to adapt to changing conditions. It recognizes that recovery can be negatively affected by cascading effects and additional hazards, emphasizing the significance of risk management to enhance resilience and sustainability practices to reconstruct the environment and revitalize the economic, social, and natural environments. Psychological and emotional recovery is recognized as vital to individuals, families, and communities.

Local governments are responsible for planning and managing a community's recovery from all disasters.[2] They shoulder the burden of preparing hazard mitigation and recovery plans, raising hazard awareness, and educating their people on resources available to enhance resilience. Even though state and federal standards exist, the local government decides whether to adopt, codify, and enforce mitigation measures. Individuals, families, and businesses will look to local leaders for support during disasters, and local governments should establish continuity of government and continuity of operations plans. They are at risk of becoming overwhelmed and will likely need assistance from state and federal offices for critical staffing and recovery expertise. A critical local asset during any biological incident is the local or county public health agency, which will have established contingency plans and can assist with coordinating medical surge when needed.

States lead, manage, and drive the overall recovery process; they coordinate recovery activities that include providing financial and technical support.[2] They serve as a conduit to local and tribal governments for federal recovery assistance programs, and they may develop programs or secure funding to finance or implement recovery projects. States can also reassign existing resources to facilitate recovery, and they play a critical role in strategic messaging to enhance public awareness. The state public health agency will play a critical role in messaging and coordinating medical assistance to the affected community. It is critical that state offices remain mission capable during a disaster, and they should also develop and maintain continuity of government and continuity of operations plans.

The federal government may use the NDRF to engage necessary and available department and agency capabilities to support local recovery efforts when a disaster exceeds the capacity of the state and tribal resources or affects federal property or national security interests.[2] Federal support is important when local and state resources are overwhelmed, especially during the early weeks following a large-scale disaster or catastrophic incident; the duration and extent of federal support will be partially determined by scale and enduring impact of the disaster. The federal government also plays a critical role in messaging to enhance public awareness about the threat and to inform stakeholders about federal grants and loans that can assist recovery efforts. The lead federal agency for coordination of health and social services during recovery is the DHHS.

Similar to ESFs in the NRF outlining federal assistance for disaster response, the NDRF identifies multiagency coordinated recovery support functions in the following areas:

- community planning and capacity building;
- economics;
- health and social services;
- housing;
- infrastructure systems; and
- natural and cultural resources.

Each annex outlines pre- and postdisaster activities as well as a list of objectives. The recovery support functions develop guidance and standard operating procedures for rapid activation to support community recovery.[2]

SUMMARY

Consequence management has historically received the least amount of planning emphasis and has not been adequately tested through robust exercises. By the nature of the problem it is complex, involves multiple agencies, and spans a considerable amount of time to exercise through response and recovery operations. The national framework documents offer a template for core capability development that can lead to readiness across a broad spectrum of potential disasters, and they can facilitate robust planning at the local level. These documents are already being used to develop plans at the state and federal levels. Unity of effort, which is critical to both effective response and recovery, can be developed through multiagency exercises. Historically, response without continuation through full recovery has been exercised. Emergency response exercises can be time compressed, but should span the full spectrum of response and recovery, so agencies will be prepared to work together when a disaster strikes.

Biological incidents are unique challenges. It is unlikely to know when a biological agent is dispersed or when the index case of a pandemic crosses a nation's border. Buildings will not be flattened, but they may be unsuitable for human occupation for an extended time, compromising critical services to the community, state, or nation. Economic impact could be significant and devastating to individuals and industries. A small focal dispersion of a biological agent could lead to broad impact with significant morbidity, mortality, and public fear if response and recovery efforts are not efficiently implemented to identify, contain, treat, protect, and clean.

Consequence management is critical to mitigating the magnitude of impact a disaster has on a community, state, and nation. History has proven that all disasters or terrorist acts cannot be prevented, but through effective consequence management the impact of both can be minimized and the terrorist's aim of maximal disruption can be defeated. Most importantly, lives can be saved.

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Chapter 5

MEDICAL MANAGEMENT OF POTENTIAL BIOLOGICAL CASUALTIES: A STEPWISE APPROACH

THEODORE J. CIESLAK, MD*

INTRODUCTION

10-STEP APPROACH TO CASUALTY MANAGEMENT
- **Step 1: Maintain a Healthy Index of Suspicion**
- **Step 2: Protect Yourself**
- **Step 3: Save the Patient's Life (Primary Assessment)**
- **Step 4. Disinfect or Decontaminate as Appropriate**
- **Step 5: Establish a Diagnosis (Secondary Assessment)**
- **Step 6: Provide Prompt Therapy**
- **Step 7: Institute Proper Infection Control Measures**
- **Step 8: Alert the Proper Authorities**
- **Step 9: Conduct an Epidemiological Investigation and Manage the Psychological Aftermath of a Biological Attack**
- **Step 10: Maintain a Level of Proficiency**

SUMMARY

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INTRODUCTION

Response to a biological attack is relatively straightforward when the etiologic agent employed is known. A larger problem arises, however, in the context of diagnostic uncertainty. In some cases, an attack may be threatened or suspected, but whether such an attack has, in fact, occurred can remain unclear. Moreover, it may be uncertain whether casualties in certain situations arise from exposure to a biological agent, a chemical or radiological agent, a naturally occurring infectious disease process, or toxic industrial exposure, or may simply reflect a heightened awareness of background disease within a community or population. Experience with West Nile virus,[1] severe acute respiratory syndrome,[2] pneumonic tularemia,[3,4] and monkeypox[5] highlight this dilemma. In each of these cases, the possibility of bioterrorism was properly raised, although each outbreak ultimately proved to have a natural origin. In some instances, proof of such an origin may be difficult or impossible to attain, providing "plausible deniability," precisely the reason some belligerents may opt to employ biological agents. This chapter provides a structured framework for dealing with outbreaks of unknown origin and etiology on the battlefield, as well as in a potential bioterrorism scenario involving military support installations or the civilian populace.

In responding to the unknown, it is helpful in many situations to employ a standardized, stepwise approach. This is especially true in the setting of a medical mass casualty event (MASCAL), where the use of such an approach (as advocated by the Advanced Trauma Life Support [ATLS] model sponsored by the American College of Surgeons[6]) is already well accepted and practiced. It is also especially true under austere or battlefield conditions. Although major theater-level and continental United States-based military medical centers (and research institutions, such as the US Army Medical Research Institute of Infectious Diseases [USAMRIID] and US Army Medical Research Institute of Chemical Defense) may possess sophisticated diagnostic and response capabilities, providers on the battlefield and at lower-role medical treatment facilities are typically required to make rapid therapeutic decisions based on incomplete information and with little immediate support. Civilian clinicians, first responders, and public health personnel practicing in rural or remote areas during a terrorist attack would face similar decision-making challenges. In the setting of a biological (or chemical or radiological) attack, similar to the setting of a MASCAL trauma event, such decisions may have life-and-death implications. In such situations, a stepwise or algorithmic approach becomes invaluable.

USAMRIID has developed a 10-step approach to managing casualties that might result from biological warfare or terrorism. Many facets of this approach may be helpful in dealing with potential chemical or radiological casualties as well. In today's complex world, it is no longer adequate for most clinicians and medical personnel to simply understand disease processes. Rather, these personnel, whether military or civilian, must have tactical, operational, and strategic knowledge of threat response—and, in fact, of disaster response in general—as it applies to weapons of mass destruction. Tactical response concerns those elements of diagnosis and treatment of specific diseases that traditionally have been the realm of the individual practitioner. Operational response can be thought of as involving the mechanisms by which the provider interacts with his or her institution (hospital, clinic, medical unit) to provide mass care during a disaster. Strategic response involves system-wide disaster preparedness and response. In a civilian setting, this includes mechanisms by which state and federal disaster response elements might become involved. Medical personnel today need to have at least a basic understanding of operational and strategic response in addition to a firm grounding in tactical medical and public health intervention. The first 7 steps of this 10-step approach deal predominately with tactical issues (ie, at the level of the individual provider). Steps 8 and 9 transition into operational and strategic response (ie, at the level of the institution and of the system, as a whole). The derivation of the 10-step approach is reported elsewhere,[7–10] and a condensed version appears in recent editions of USAMRIID's *Blue Book*.[11] It is expanded upon here.

10-STEP APPROACH TO CASUALTY MANAGEMENT

Step 1: Maintain a Healthy Index of Suspicion

In the case of chemical warfare or terrorism, the intentional nature of an attack is often evident. In this case, victims would likely be tightly clustered in time and space; they would succumb in close proximity (both temporally and geographically) to a dispersal device. Complicating the discovery of the intentional nature of a biological attack, however, is the fact that biological agents possess inherent incubation periods, while conventional, chemical, and nuclear weapons do not. These incubation periods, typically of several days

(but up to several weeks in the case of agents such as *Coxiella burnetii* and the *Brucellae*), allow for the wide dispersion of victims in time and space. Additionally, they make it likely that the first responder to a biological attack would not be the firefighter, police officer, paramedic, or other traditional first responder, but rather primary care providers, hospital emergency departments, and public health officials. In such circumstances, maintaining a healthy index of suspicion is imperative.

In some instances, maintaining an index of suspicion might be simplified by the fact that diseases caused by biological agents may present with specific characteristic clinical findings, which allow for a very limited differential diagnosis. The hallmark of inhalational anthrax is a widened mediastinum, a clinical finding seen in few naturally occurring conditions. With botulism, the hallmark presentation is that of a descending, symmetric, flaccid paralysis. Whereas an individual patient with flaccid paralysis might prompt consideration of disorders such as Guillain-Barré syndrome, Eaton-Lambert syndrome, poliomyelitis, and myasthenia gravis, the near-simultaneous presentation of multiple patients with flaccid paralysis should quickly lead one to a diagnosis of botulism. Similarly, patients with plague and melioidosis may exhibit hemoptysis in the later stages of illness. Such a finding is uncommon among previously healthy individuals, but can be caused by tuberculosis, staphylococcal and *Klebsiella* pneumonia, carcinoma, and trauma. Multiple patients with hemoptysis, however, should prompt consideration of a plague or melioidosis diagnosis. Smallpox is characterized by a very unique exanthem, perhaps evocative of *Varicella* or syphilis in its earliest stages, but readily distinguishable from these entities as it progresses.

Yet, by the time each of these characteristic findings develops, treatment is less likely to be effective. Therapy is thus best instituted during the incubation or prodromal phases of these diseases if it is to be beneficial. Unfortunately, during their prodromes, these diseases are likely to appear as undifferentiated febrile illnesses, difficult, if not impossible, to distinguish from myriad other common infectious diseases. Similarly, many other diseases potentially arising from a biological attack (such as tularemia, brucellosis, melioidosis, Q fever, and Venezuelan equine encephalitis) may appear simply as undifferentiated febrile illnesses throughout their course. Prompt diagnosis and targeted therapy is thus possible only with a very high index of suspicion.

Epidemiological clues can lead a clinician to suspect that a disease outbreak may have been intentional (Exhibit 5-1).[12] Large numbers of victims tightly clustered in time and space, or limited to a discrete population, should raise suspicion. Similarly, unexpected deaths and cases of unexpectedly severe illness merit concern. An outbreak of a disease not typically seen in a specific geographic location, in a given age group, or during a certain season, likewise warrants further investigation. Simultaneous outbreaks of a disease in noncontiguous areas should prompt one to consider an intentional release, as should simultaneous or sequential outbreaks of different diseases in the same locale. Even a single case of rare disorders, such as anthrax or certain viral hemorrhagic fevers (Ebola, Marburg, Lassa, and many others) would be suspicious, and a single case of smallpox, because it no longer occurs naturally, would almost certainly represent an intentional release. The presence of dying animals (or the simultaneous occurrence of zoonotic disease outbreaks among humans and animals) might provide evidence of an unnatural aerosol release. Evidence of a disparate attack rate between those known to be indoors and outdoors at a given time should also be sought and evaluated.

EXHIBIT 5-1

EPIDEMIOLOGICAL CLUES TO A BIOTERRORIST ATTACK

- Presence of an unusually large epidemic
- High infection rate
- Disease limited to a discrete population
- Unexpected severity of disease
- Evidence of an unusual route of exposure
- Disease in an atypical geographic locale
- Disease occurring outside normal transmission seasons
- Disease occurring in the absence of usual vector
- Simultaneous outbreaks of multiple diseases
- Simultaneous occurrence of human and zoonotic disease
- Unusual organism strains
- Unusual antimicrobial sensitivity patterns
- Disparity in attack rates among persons indoors and outdoors
- Terrorist claims
- Intelligence reports
- Discovery of unusual munitions

Data source: Pavlin JA. Epidemiology of bioterrorism. *Emerg Infect Dis*. 1999;5:528–30.

Finally, intelligence reports, terrorist claims, and the discovery of aerosol spray devices would obviously lend credence to the theory that a disease outbreak was of sinister origin.

On the modern battlefield, an array of developing technology is available to assist clinicians, preventive medicine and chemical corps personnel, operators, and commanders in maintaining their index of suspicion through early "stand-off" detection of biological threats. The Portal Shield is the Department of Defense's (DoD's) first automated biological detection system, and was designed to provide fixed-site protection to air and port facilities. Portal Shield is equipped with modular sensors capable of simultaneously assaying for eight different agents and providing presumptive identification within about 25 minutes. The Biological Integrated Detection System, a system mounted on a high-mobility multipurpose wheeled vehicle, is equipped with samplers, an aerodynamic particle sizer, a flow cytometer, and a chemical biological mass spectrometer. The Joint Biological Point Detection System integrates into the M31A2 Biological Integrated Detection System platform (Figure 5-1) to permit rapid, real-time detection of 10 separate biological threat agents on the battlefield; the system is capable of definitively identifying biowarfare threat agents within 18 minutes. The Joint Biological Agent Identification and Diagnostic Systems (JBAIDS) is a reusable, portable, and modifiable biological agent identification and diagnostic system capable of rapid, reliable, and simultaneous identification of multiple biological agents and other pathogens of operational concern. The JBAIDS anthrax, tularemia, plague, and Q fever detection systems are cleared by the Food and Drug Administration (FDA) for diagnostic use. Until these technologies are refined, validated, and made widely available, though, those tasked with responding to an attack must rely on clinical, epidemiological, and intelligence clues to maintain their index of suspicion.



Figure 5-1. The Biological Integrated Detection System (BIDS) is a semi-automated biological agent detection/identification suite mounted on a dedicated heavy high mobility multipurpose wheeled vehicle. The system uses multicomplimentary bio-detection technologies.

Step 2: Protect Yourself

Providers who themselves become casualties are of little use to their patients. Before approaching casualties of biological or chemical warfare or victims of a potential terrorist attack, clinicians should be familiar with basic means of self-protection. Such protective measures generally fall into one of three categories: (1) physical protection, (2) chemical protection, and (3) immunologic protection. Under a given set of circumstances, clinicians and laboratory personnel might appropriately avail themselves of one or more of these forms of protection.

Physical Protection

Since the beginning of modern gas warfare on the battlefields near Ypres, Belgium, in 1915, physical protection during military operations has involved gas masks and, more recently, charcoal-impregnated chemical protective overgarments. Although military-style protective clothing and masks were designed with chemical agent protection in mind, they are capable of offering protection against biological agents as well. Although some countries have advocated the issuance of military-style protective masks and ensembles to civilians (eg, the Israeli government has issued masks to its general populace), such items, even if offered, would likely be unavailable to civilians at the precise moment of agent release; the unannounced release of odorless and colorless biological agents by belligerents or terrorists would afford no opportunity to don protective gear, even if it were available. Furthermore, misuse of protective equipment in the past has led to fatalities, including cases of infants and adults suffocating in protective ensembles.[13,14] Although military masks such as the M40/42, M45, and M50 series provide ample protection against inhalation hazards posed by chemical and biological weapons as well as against radioactive dust particles, they add heat stress and are potentially mission-degrading. Moreover, a simple surgical mask will usually afford adequate protection against inhalation of infectious aerosols of virtually any of the biological agents typically mentioned in a terrorism context. An important exception might be smallpox, in which case a high-efficiency

particulate air (HEPA) filter mask would be ideal. With the exception of smallpox, pneumonic plague, and certain viral hemorrhagic fevers, the agents in the Centers for Disease Control and Prevention's (CDC's) categories A and B (Exhibit 5-2) are not contagious via the respiratory route. Respiratory protection is thus necessary when operating in an area of primary release, but would not be required in most patient-care settings (see step 7).

Chemical Protection

During Operations Desert Shield and Desert Storm, tens of thousands of US troops were given pyridostigmine under an emergency-use authorization, and in early 2003, the FDA gave its final approval for the use of pyridostigmine bromide as preexposure prophylaxis against intoxication with soman, an organophosphate-based chemical nerve agent. It is conceivable, given credible and specific intelligence, that similar strategies might be employed against biological weapons. For example, if a specific terrorist group possessing a specific weaponized agent were known to be operating in a given locale, public health authorities might conceivably contemplate the widespread distribution of an appropriate prophylactic antibiotic. Obviously, the opportunities to employ such a strategy are likely to remain few and far between, and the logistics of doing so would be exceedingly difficult in a civilian setting.

Immunologic Protection

For the near future, active vaccination is likely to provide one of the most practical methods for administering preexposure prophylaxis against biological attack. In the military, decisions regarding vaccination policy are typically made through the office of the Assistant Secretary of Defense for Health Affairs, with input from high-level military medical, public health, and intelligence sources. The decision to offer a specific vaccine in a specific circumstance is a complex one that must take into account a careful risk-benefit calculation. During Operations Desert Shield and Desert Storm, some 150,000 service members received at least one dose of anthrax vaccine, while about 8,000 received a botulinum toxoid vaccine. Since 1998, the US military has intermittently employed force-wide anthrax vaccination, and since 2003 has administered smallpox vaccine to deploying troops and certain medical response teams.

In a civilian counter-terrorism context, the decision to employ a specific vaccine is even more difficult and complex. Factors that would influence a decision by public health officials to recommend vaccination include intelligence (eg, how likely or plausible is an attack? How imminent is the threat? How specific is the threat?), vaccine safety, vaccine

EXHIBIT 5-2

CRITICAL AGENTS FOR HEALTH PREPAREDNESS

Category A*	Category B†	Category C‡
• Variola virus • *Bacillus anthracis* • *Yersinia pestis* • Botulinum toxin • *Francisella tularensis* • Filoviruses and arenaviruses	• *Coxiella burnetii* • *Brucellae* • *Burkholderia mallei* • *Burkholderia pseudomallei* • Alphaviruses • Certain toxins (ricin, staphylococcal enterotoxin B, trichothecenes) • Food safety threat agents (Salmonellae, *Escherichia coli* O157:H7) • Water safety threat agents (*Vibrio cholerae*, etc)	Other biological agents that may emerge as future threats to public health, such as: • Nipah virus • Hantaviruses • Yellow fever virus • Drug-resistant tuberculosis • Tick-borne encephalitis

*Agents with high public health impact requiring intensive public health preparedness and intervention.
†Agents with a somewhat lesser need for public health preparedness.
‡Other biological agents that may emerge as future threats to public health.
Data source: Centers for Disease Control and Prevention. Biological and chemical terrorism: strategic plan for preparedness and response. *MMWR*. 2000;49(RR-04):1–14.

availability, disease consequences (ie, is the threat from a lethal agent or from an incapacitant?), and the availability of postexposure prophylaxis or therapy. Recently, civilian public health and policy planners have given extensive consideration to the widespread distribution of anthrax and smallpox vaccines.

Anthrax. Anthrax Vaccine, Adsorbed (AVA, BioThrax; Emergent BioSolutions, Lansing MI) is a fully licensed product, approved by the FDA in 1970. The vaccine consists of a purified preparation of protective antigen, a potent immunogen necessary for entry of key anthrax toxin components (lethal and edema factors) into mammalian cells. Administered alone, protective antigen is nontoxic. In a large controlled trial, AVA was effective in preventing cutaneous anthrax among textile workers.[15] Based on an increasing amount of animal data, there is every reason to believe that this vaccine is quite effective at preventing inhalational anthrax as well.[16] Moreover, well over 20 clinical studies, surveys, and reports now attest to the safety of AVA,[17,18] and the FDA has reaffirmed the vaccine as being safe and effective in light of those studies.[19] Nonetheless, although widespread use of AVA has occurred within the US military (as of January 2014, more than 12.1 million doses of AVA had been given to more than 2.4 million service members), logistical and other considerations make large-scale civilian vaccination impractical at present. The vaccine is licensed as a five-dose series, given at 0 and 4 weeks, and at 6, 12, and 18 months. Yearly boosters are recommended for those at ongoing risk of exposure. Further complicating any potential civilian anthrax vaccination strategy is the fact that AVA is approved by the FDA only for individuals 18 to 65 years old. Although a large-scale preexposure offering of AVA to the general public might thus be problematic, some have recommended that a three-dose series of AVA (given at time zero and at 2 and 4 weeks after the initial dose), combined with 60 days of antibiotics under an investigational new drug (IND) protocol or emergency use authorization, might be an acceptable alternative to longer (60–100 days) antibiotic courses alone for postexposure prophylaxis against inhalational anthrax.[20] This recommendation was based on nonhuman primate challenge studies; no human studies currently exist to support such a strategy, and AVA is not licensed by the FDA for postexposure prophylaxis or therapy.

Smallpox. Widespread vaccination against smallpox is equally controversial and problematic. Nonetheless, in 2002, President George W. Bush announced a plan to vaccinate selected American healthcare workers and military personnel. Within the DoD, service members deploying to locations thought at risk for biological attack and members of designated smallpox epidemiological and clinical response teams were selected for vaccination. The program includes prevaccination screening to exclude members with vaccine contraindications or household contacts at risk, instruction on vaccine site care and potential complications, and mandatory follow-up. As of January 10, 2014, over 2.4 million military response team members, hospital workers, and operational forces had been vaccinated, with one death occurring due to a lupus-like illness. Although the emergence of myopericarditis (there were 161 confirmed, suspected, or probable cases among 1.4 million vaccinees as of January 2008) as a complication of vaccination[21] led to a revision of prevaccine screening (candidates with multiple cardiac risk factors are now excluded), rates of other adverse reactions were low. Cases of autoinoculation or transmission to household and other contacts have been rare.[22–24] One case of progressive vaccinia occurred in a primary vaccine recipient,[25] and three cases of eczema vaccinatum occurred among contacts of vaccinees.[26,27] No cases of fetal vaccinia have been reported. Vaccinia immune globulin was required on only seven occasions, to treat ocular vaccinia,[28] progressive vaccinia,[26] eczema vaccinatum,[27,28] and as prophylaxis for a vaccinated patient who sustained large burn wounds. The success of this smallpox immunization program suggests that mass vaccination can be accomplished with greater safety than previously thought possible.[29]

Although universal civilian vaccination was not recommended under President Bush's plan, the possibility of a future strategy calling for such recommendations was allowed for, and provisions were made to provide smallpox vaccine to those members of the general public who specifically requested it. The wisdom of widespread civilian vaccination is difficult to assess. Most medical decisions involve a risk-benefit analysis on the part of the responsible clinician. In the case of smallpox vaccination, the risks are well known, and they are significant.[30,31] The benefits, however, are far less certain; although the global eradication of smallpox surely ranks among the greatest public health accomplishments of recent history and the wisdom of vaccination with live vaccines went unquestioned during the era of endemic smallpox, the likelihood of contracting smallpox today via a terrorist attack is unknown and likely miniscule for the average civilian. In this regard, the risk-benefit calculation is not based on medical considerations, but rather on intelligence estimates to which few are privy.

Despite these concerns, a prerelease mass vaccination program for the general population may be the most effective countermeasure to the terror threat

posed by smallpox. By inducing individual and herd immunity and by obviating the extreme difficulty of conducting postrelease vaccine and quarantine efforts, a program involving the resumption of universal smallpox vaccination possesses distinct advantages over other response plans. However, such an approach is hampered not only by the unknown risk of a smallpox release, but also by safety and logistics issues.[32,33]

A large number of persons are at risk for severe vaccine reactions today compared to the previous era of routine civilian smallpox vaccination, which ended in 1972. This increase in risk is due to the presence in the population of a large number of persons with compromised immunity associated with human immunodeficiency virus and with advances in immunosuppressive therapy and bone marrow and solid organ transplantation. This phenomenon raises concern about the safety and risk-benefit ratio of any preexposure vaccination program.[34] Similarly, the occurrence of rare but severe smallpox vaccine complications in otherwise healthy recipients could result in morbidity and mortality that would be unacceptable in times of low risk. Risk analysis favors prerelease mass vaccination of the general population only if the probability of a large-scale attack is high. Prerelease mass vaccination of healthcare workers might again be contemplated in the future, owing to the risk of exposure while caring for patients, and the value of keeping healthcare workers healthy and functioning in the setting of an epidemic.[35]

The smallpox vaccine currently employed in the United States is ACAM2000 (Acambis Inc, Cambridge, MA), which uses modern cell-culture-based production of vaccinia, an orthopoxvirus closely related to variola. ACAM2000 was licensed by the FDA in 2007, and replaced Dryvax (Wyeth Laboratories, Marietta, PA), a preparation derived from the harvested lymph of inoculated calves, in 2008. It is unlikely that this will significantly diminish the risk of adverse reactions, however, as the new vaccine employs the same live strain of vaccinia virus. The vast majority of adverse reactions to current vaccinia-containing vaccines derive from the live nature of the virus rather than the method of preparation.

The CDC controls release of civilian ACAM2000 stocks and conditions for release have been established.[36] The current CDC smallpox response strategy is based on preexposure vaccination of carefully screened first responders and members of epidemiological and clinical response teams. CDC plans also provide for a program to treat certain severe complications of vaccination using vaccinia immune globulin under an IND protocol, as well as for compensation of persons experiencing such complications, through the establishment of a smallpox vaccine injury compensation program.[37]

The CDC's response plan calls for "ring vaccination" after a smallpox release: identification and isolation of cases, with vaccination and active surveillance of contacts. Mass vaccination would be reserved for those instances when the number or location of cases renders the ring strategy inefficient, or when the risk of additional virus release is high.[38] Although ring vaccination was successful historically (in the setting of herd immunity), mathematical models predict that this strategy may be problematic when applied to large or multifocal epidemics today.[39] Furthermore, there is controversy among experts regarding the predicted benefit of postrelease mass vaccination due to lack of herd immunity, a highly mobile population, a relatively long incubation period, and the difficulties associated with prompt implementation of quarantine and mass vaccination.[40,41] Finally, it should be kept in mind that vaccination is but one component of a multifaceted response, which should also include farsighted planning and logistical preparation, risk communication, surveillance, treatment, isolation, and quarantine.

Other Agents. Few authorities, either military or civilian, have advocated widespread vaccination against potential agents of bioterrorism other than anthrax and smallpox, and the implementation of any such strategy would currently be problematic. A vaccine against plague, previously licensed in the United States, is currently out of production. It required a three-dose primary series followed by annual boosters. Moreover, it was licensed only for persons 18 to 61 years old. Finally, although reasonably effective against bubonic plague and widely employed by the DoD to protect against endemic disease, it probably afforded little protection against pneumonic plague, the form of disease likely to be associated with warfare or terrorism. A vaccine against one specific viral hemorrhagic fever, namely yellow fever, is widely available, although its causative virus is not regarded as a significant weaponization threat by most policymakers and health officials. Again, while the US military has administered yellow fever vaccine to large numbers of troops, it does so to guard against endemic disease, rather than a bioweapon threat. Additionally, a vaccine against Q fever (Q Vax, CSL Ltd, Victoria, Australia) is licensed in Australia. Although this vaccine might conceivably prove a useful addition to the military biodefense armamentarium, the self-limited nature of Q fever makes it unlikely that widespread use of this vaccine would be contemplated for the general public. Numerous research efforts are aimed at developing improved next-generation vaccines against anthrax, smallpox, and plague. Similarly, vaccines effective against tularemia,

brucellosis, botulism, the equine encephalitides, staphylococcal enterotoxins, ricin, and several viral hemorrhagic fevers, as well as other potential agents of bioterrorism, are in various stages of development.[42] Investigational vaccines against tularemia, botulism, the equine encephalitides (especially Venezuelan equine encephalitis), staphylococcal enterotoxin B, Q fever, and other agents, have been used under IND protocols to protect scientists studying these agents.

Step 3: Save the Patient's Life (Primary Assessment)

Once self-protective measures are implemented, the clinician can approach the MASCAL scenario and begin assessing patients (the "primary survey," in keeping with ATLS guidelines[6]). This initial assessment is intended to be brief and its purpose limited to the discovery and treatment of those conditions presenting an immediate threat to life or limb. Biological (or chemical) warfare victims may also have conventional injuries; attention should thus be focused at this point on maintaining a patent airway and providing for adequate breathing and circulation. The need for decontamination and administration of antidotes for rapid-acting chemical agents (nerve agents and cyanide) should be determined at this time. An "ABCDE" algorithm aids the clinician in recalling the specifics of the primary assessment. "A" stands for airway, which should be evaluated for the presence of conventional injury, but should also be examined because exposure to certain chemical agents (such as mustard, lewisite, or phosgene) can damage the airway. "B" denotes breathing; many agents of biological (and chemical) terrorism may cause the patient to experience respiratory difficulty. Examples include anthrax, plague, tularemia, botulism, Q fever, the staphylococcal enterotoxins, and ricin, as well as cyanide, nerve agents, and phosgene. "C" denotes circulation, which may be compromised due to conventional or traumatic injuries sustained during a MASCAL event, but may also be involved in the septic shock associated with plague and in the circulatory collapse associated with the viral hemorrhagic fevers. "D" refers to disability, specifically, neuromuscular disability. Note that botulism and nerve agent exposures are likely to present with a preponderance of neuromuscular symptomatology. Finally, "E" refers to exposure. In a MASCAL setting, this serves as a reminder to remove the victim's clothing to perform a more thorough secondary assessment. It is here that one considers the need for decontamination and disinfection.

Step 4: Disinfect or Decontaminate as Appropriate

Once patients have been stabilized, decontamination can be accomplished, where appropriate. On the battlefield, considerable mature military doctrine drives decontamination efforts, which are carried out by unit personnel, guided or assisted by specific, highly trained Chemical Corps decontamination units. It should be pointed out, however, that decontamination, in the classical sense, may not be necessary after a biological attack (the same cannot always be said after a chemical attack). This is due, again, to the inherent incubation periods of biological agents. Because victims will not typically become symptomatic until several days after exposure to such agents, they are likely to have bathed and changed clothing several times before presenting for medical care, thus effectively accomplishing self-decontamination. Exceptions might include personnel directly exposed to an observed attack or persons encountering a substance in a threatening letter, where common sense might dictate topical disinfection. Even in these situations, bathing with soap and water and conventional laundry measures would likely be adequate. Moreover, it should be kept in mind that situations such as the case of the threatening letter represent crime scenes. Any medical interest in disinfection must be weighed against law enforcement concerns regarding preservation of vital evidence, which can be destroyed through hasty and ill-considered attempts at decontamination. Furthermore, significant psychological stress has been caused by unnecessary, costly, and resource-intensive attempts at decontamination in the past.[43] Some of these attempts have involved forced disrobing and showering in public streets; to avoid such problems, the following measured responses should be considered.[44]

The Announced Threat (or Presumed Hoax). The need to preserve evidence, and maintain a chain-of-custody when handling that evidence, is an important consideration at any crime scene. Although human and environmental health protection concerns take precedence over law enforcement procedures, threat and hoax scenarios nonetheless require the early involvement of law enforcement personnel and a respect for the need to maintain an uncompromised crime scene. Decontamination or disinfection is not typically necessary.

The Telephoned Threat or the "Empty Letter." In the majority of cases involving a telephoned threat, no delivery device or package is located. If a device is found or a threat is subsequently deemed credible, public health authorities should contact potentially exposed individuals, obtain appropriate information, and consider instituting prophylaxis or therapy. An envelope containing nothing other than a written threat poses little risk and should be handled in the same manner as a telephoned threat. Because the

envelope constitutes evidence in a crime, however, further handling should be left to law enforcement professionals. In these cases, no decontamination is typically necessary, pending results of the legal and public health investigation.

The Suspicious Package. When a package is discovered and found to contain powder, liquid, or other physical material, response should be individualized. In most cases, the package should not be disturbed further, the room should be vacated, additional untrained persons should be prohibited from approaching the scene and from handling the package or its contents, and law enforcement and public health officials should again be promptly notified. Persons who have come in contact with contents should remove clothing as soon as practical and seal it in a plastic bag. Victims should then wash with soap and water[45] and, in most cases, may be sent home after adequate instructions for follow-up are provided and contact information obtained. In general, antibiotic prophylaxis would not be necessary before the preliminary identification of package contents by a competent laboratory, although decisions to provide or withhold postexposure prophylaxis are best made after consultation with public health authorities. Floors, walls, and furniture would not require decontamination before laboratory analysis is completed. Nonporous contaminated personal items, such as eyeglasses and jewelry, may be washed with soap and water or immersed in 0.5% hypochlorite (household bleach diluted tenfold) if a foreign substance has contacted the items.

The Delivery Device. If an aerosol delivery device or other evidence of a credible aerosol threat is discovered, the room (and potentially the building) should be evacuated. Law enforcement and public health personnel should be notified immediately and further handling of the device left to personnel with highly specialized training, such as the Army's 22nd and 110th Chemical Battalions (Technical Escort Units), the Marine Corps Chemical-Biological Incident Response Force (CBIRF), or the Federal Bureau of Investigation's Hazardous Materials Response Unit. Contact information should be obtained from potential victims and detailed instructions provided. Clothing removal, soap and water showering, and decontamination of personal effects should be accomplished as above (the CBIRF brings with it extensive decontamination capabilities). Decisions regarding institution of empiric postexposure prophylaxis pending determination of the nature of the threat and identification of the involved biological agents should again be left to local and state public health authorities. In providing a reasoned and measured response to each situation, public health and law enforcement personnel can assist in minimizing the disruption and cost associated with large-scale decontamination, costly hazardous materials unit involvement, and broad institution of therapeutic interventions, and can help avoid widespread public panic.

Step 5: Establish a Diagnosis (Secondary Assessment)

Once decontamination has been considered, and accomplished as warranted, the clinician may perform a more thorough and targeted assessment aimed at establishing a diagnosis (the ATLS "secondary survey"). The thoroughness and accuracy with which one establishes this diagnosis will vary depending upon the circumstances the clinician finds him- or herself in. At robust roles of care (Role 4), the clinician may well have access to infectious disease and microbiology professionals, as well as to sophisticated diagnostic assays. Under such circumstances, it may be possible to arrive at a definitive microbiologic diagnosis fairly promptly. On the other hand, it is equally conceivable that the primary care provider, practicing at lower roles of care (Roles 1 to 3) or in more austere circumstances, may need to intervene promptly based on limited information and without immediate access to subspecialty consultation. Even in such cases, however, reasonable care can be instituted based simply on a syndromic diagnosis. An "AMPLE" (A: allergies, arthropod exposures; M: medications [as well as military occupational specialty and mission-oriented protective posture status]; P: past illnesses and vaccinations; L: last meal; E: environment) history may aid in establishing this diagnosis. A brief but focused physical examination, even one performed by inexperienced practitioners, can, at a minimum, reveal whether a victim of a biological or chemical attack exhibits primarily respiratory, neuromuscular, or dermatologic signs, or suffers simply from an undifferentiated febrile illness. By placing patients into one of these broad syndromic categories, empiric therapy can be initiated (see step 6); such empiric therapy can be refined and tailored once more information becomes available.[46,47]

When the situation permits, laboratory studies should be obtained to aid in later definitive diagnosis (Exhibit 5-3). On the battlefield, samples obtained at lower echelons would normally be submitted to the local clinical laboratory and, from there, through clinical laboratory channels to the 1st Area Medical Laboratory (AML). The AML is a theater-level tactical laboratory with very robust scientific capabilities, including the ability to rapidly identify biological, chemical, and radiological threat agents, as well as endemic, occupational, and environmental health hazards. The AML also has "reach-back" ability and works closely

EXHIBIT 5-3

SAMPLES TO CONSIDER OBTAINING FROM POTENTIAL BIOWARFARE OR BIOTERRORISM VICTIMS*

- Complete blood count
- Arterial blood gas
- Nasal swabs for culture and PCR
- Blood for bacterial culture and PCR
- Serum for serologic studies
- Sputum for bacterial culture
- Blood and urine for toxin assay
- Throat swab for viral culture, PCR, and ELISA
- Environmental samples

*This list is not all-inclusive, nor is it meant to imply that every sample should be obtained from every patient. In general, laboratory sampling should be guided by clinical judgment and the specifics of the situation. This is a list of samples to consider obtaining in situations where the nature of an incident is unclear and empiric therapy must be started before definitive diagnosis.
ELISA: enzyme-linked immunosorbent assay
PCR: polymerase chain reaction

with national laboratories at USAMRIID and the US Army Medical Research Institute of Chemical Defense in Maryland.

Step 6: Provide Prompt Therapy

Once a diagnosis (whether definitive or syndromic) is established, prompt therapy must be provided. In the cases of anthrax and plague, in particular, survival is directly linked to the speed with which appropriate therapy is instituted. A delay of more than 24 hours in the treatment of either disease leads to a uniformly grim prognosis. When the identity of a bioterrorist agent is known, the provision of proper therapy is straightforward (Table 5-1). When a clinician is faced with multiple victims and the nature of the illness is not known, however, empiric therapy must be instituted. Guidelines for providing empiric therapy in such situations have been published, and an algorithmic approach to syndromic diagnosis and empiric therapy has been developed (Figure 5-2). Doxycycline, ciprofloxacin, or levofloxacin should be administered empirically to patients with significant respiratory symptoms when exposure to a biological attack is considered a possibility.

Step 7: Institute Proper Infection Control Measures

The clinician must practice proper infection control procedures to ensure that contagious diseases are not propagated among patients. The majority of biological threat agents are not contagious. Among these are the causative agents of anthrax, tularemia, brucellosis, Q fever, the alphaviral equine encephalitides, glanders, melioidosis, and many others, including all of the toxins. Standard precautions alone suffice, in most cases, when caring for victims of such diseases.[48] More stringent transmission-based precautions should be applied in certain circumstances. Three subcategories of transmission-based precautions exist. Droplet precautions are required to manage victims of pneumonic plague. Ordinary surgical masks are a component of proper droplet precautions and constitute adequate protection against acquisition of plague bacilli by the aerosol route. Contact precautions should be employed when managing certain viral hemorrhagic fever patients. In theory, these would be adequate for managing even Ebola victims given the transmission of this disease through infected blood and body fluids. Recent experience with Ebola in West Africa, however, illustrates the ease with which such precautions might be compromised. Given the prodigious amounts of body fluids (emesis and diarrhea) produced by these patients, the very low infectious inoculum of Ebola, and the propensity for hemorrhagic sputum to be aerosolized during coughing, the CDC now recommends that both contact and droplet precautions be employed when managing Ebola victims. Airborne precautions, ideally including an N-95 HEPA-filter mask, should be used when caring for smallpox victims. A summary of hospital infection control precautions as they apply to victims of biological terrorism is presented in Exhibit 5-4.

Step 8: Alert the Proper Authorities

As soon as it is suspected that a case of disease might be the result of exposure to biological or chemical agents, the proper authorities must be alerted so that appropriate warnings may be issued and outbreak-control measures implemented. On the battlefield and in other military settings, the command must be notified immediately. It is similarly important to notify preventive medicine officials, as well as chemical corps and laboratory personnel. Early involvement of preventive medicine personnel ensures that an epidemiological investigation is begun promptly (see step 9) and that potential victims (beyond the index cases) are identified and treated early, when such treatment is most likely to be beneficial. Similarly, early notification of Army chemical corps personnel allows for battlefield surveillance, detection, and

TABLE 5-1

RECOMMENDED THERAPY OF AND PROPHYLAXIS AGAINST DISEASES CAUSED BY CATEGORY A BIOTHREAT AGENTS

Condition	Adults	Children
Anthrax, inhalational, therapy* (patients who are clinically stable after 14 days can be switched to a single oral agent [ciprofloxacin or doxycycline] to complete a 60-day course†)	Ciprofloxacin 400 mg IV q12h **OR** Levofloxacin 500 mg IV q24h **OR** Doxycycline 100 mg IV q12h **AND** Clindamycin‡ 900 mg IV q8h **AND** Penicillin G§ 4 mil U IV q4h **AND CONSIDER** Raxibacumab 40 mg/kg IV	Ciprofloxacin 10–15 mg/kg IV q12h **OR** Levofloxacin 8 mg/kg IV q12h **OR** Doxycycline 2.2 mg/kg IV q12h **AND** Clindamycin‡ 10–15 mg/kg IV q8h **AND** Penicillin G§ 400–600 k U/kg/d IV × q4h **AND CONSIDER** Raxibacumab IV (> 50 kg: 40 mg/kg; 15–50 kg: 60 mg/kg; < 15 kg: 80 mg/kg)
Anthrax, inhalational, postexposure prophylaxis (60-day course†)	Ciprofloxacin 500 mg PO q12h **OR** Levofloxacin 500 mg PO q24h **OR** Doxycycline 100 mg PO q12h	Ciprofloxacin 10–15 mg/kg PO q12h **OR** Levofloxacin 8 mg/kg PO q12h **OR** Doxycycline 2.2 mg/kg PO q12h
Anthrax, cutaneous in setting of terrorism, therapy¥	Ciprofloxacin 500 mg PO q12h **OR** Levofloxacin 500 mg PO q24h **OR** Doxycycline 100 mg PO q12h	Ciprofloxacin 10–15 mg/kg PO q12h **OR** Levofloxacin 8 mg/kg PO q12h **OR** Doxycycline 2.2 mg/kg PO q12h
Plague, therapy	Gentamicin 5 mg/kg IV qd **OR** Doxycycline 100 mg IV q12h **OR** Ciprofloxacin 400 mg IV q12h **OR** Levofloxacin 500 mg IV q24h	Gentamicin 2.5 mg/kg IV q8h **OR** Doxycycline 2.2 mg/kg IV q12h **OR** Ciprofloxacin 15 mg/kg IV q12h **OR** Levofloxacin 8 mg/kg IV q12h
Plague, prophylaxis	Doxycycline 100 mg PO q12h **OR** Ciprofloxacin 500 mg PO q12h **OR** Levofloxacin 500 mg PO q24h	Doxycycline 2.2 mg/kg PO q12h **OR** Ciprofloxacin 20 mg/kg PO q12h **OR** Levofloxacin 8 mg/kg PO q12h
Tularemia, therapy	Gentamicin 5 mg/kg IV qd **OR** Doxycycline 100 mg IV q12h **OR** Ciprofloxacin 400 mg IV q12h	Gentamicin 2.5 mg/kg IV q8h **OR** Doxycycline 2.2 mg/kg IV q12h **OR** Ciprofloxacin 15 mg/kg IV q12h
Tularemia, prophylaxis	Doxycycline 100 mg PO q12h **OR** Ciprofloxacin 500 mg PO q12h	Doxycycline 2.2 mg/kg PO q12h **OR** Ciprofloxacin 20 mg/kg PO q12h
Smallpox, therapy	Supportive care	Supportive care
Smallpox, prophylaxis	Vaccination may be effective if given within the first several days after exposure.	Vaccination may be effective if given within the first several days after exposure.
Botulism, therapy	Supportive care; antitoxin may halt the progression of symptoms but is unlikely to reverse them.	Supportive care; antitoxin may halt the progression of symptoms but is unlikely to reverse them.
Viral hemorrhagic fevers, therapy	Supportive care; ribavirin may be beneficial in select cases.	Supportive care; ribavirin may be beneficial in select cases.

*In a mass casualty setting, where resources are severely constrained, oral therapy may need to be substituted for the preferred parenteral option.
†Assuming the organism is sensitive, children may be switched to oral amoxicillin (80 mg/kg/d× q8h) to complete a 60-day course. We recommend that the first 14 days of therapy or postexposure prophylaxis, however, include ciprofloxacin, levofloxacin, or doxycycline regardless of age. A three-dose series of Anthrax Vaccine Adsorbed may permit shortening of the antibiotic course to 30 days.
‡Rifampin or clarithromycin may be acceptable alternatives to clindamycin as drugs that target bacterial protein synthesis. If ciprofloxacin or another quinolone is employed, doxycycline may be used as a second agent, as it also targets protein synthesis.
§Ampicillin, imipenem, meropenem, or chloramphenicol may be acceptable alternatives to penicillin as drugs with good central nervous system penetration.
¥10 days of therapy may be adequate for endemic cutaneous disease. A full 60-day course is recommended in the setting of terrorism, however, because of the possibility of a concomitant inhalational exposure.
IV: intravenous; PO: per os (by mouth)

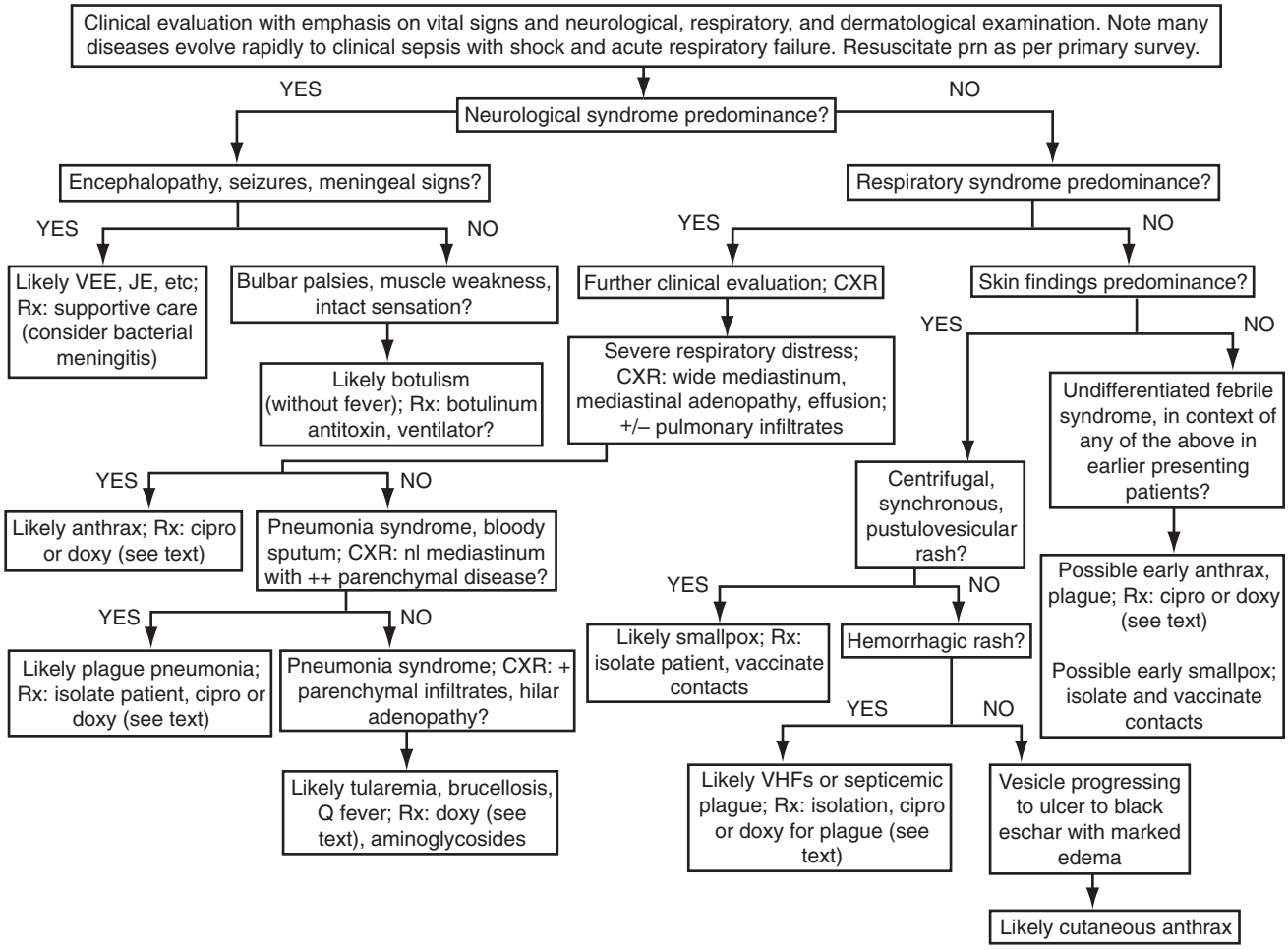


Figure 5-2. An empiric and algorithmic approach to the diagnosis and management of potential biological casualties.
cipro: ciprofloxacin; CXR: chest X-ray; doxy: doxycycline; JE: Japanese encephalitis; nl: normal limits; prn: as needed; Rx: prescription; VEE: Venezuelan equine encephalitis; VHF: viral hemorrhagic fever; +: positive finding; ++: strongly positive finding; +/–: with or without finding
Adapted with permission from Henretig FM, Cieslak TJ, Kortepeter MG, Fleisher GR. Medical management of the suspected victim of bioterrorism: an algorithmic approach to the undifferentiated patient. *Emerg Med Clin North Am.* 2002;20:351–364.



Figure 5-3. The M93 "Fox" nuclear, biological, and chemical reconnaissance vehicle.

delineation of the limits of contamination. Using M93 "Fox" or M1135 Stryker (Figure 5-3) nuclear, biological, chemical reconnaissance vehicles, these personnel can collect soil, water, and vegetation samples, mark areas of contamination, and transmit data to commanders in real time. Finally, notifying laboratory personnel not only permits them to focus their efforts at diagnosis, but also allows them to take necessary precautions.

In a civilian terrorism response scenario, notification of a suspected biological, chemical, or radiological attack would typically be made through local or regional health department channels. In the United States, a few larger cities have their own health departments. In most areas, though, the county represents the lowest jurisdiction at which an independent health department exists. In some rural areas lacking county

EXHIBIT 5-4

CONVENTIONAL INFECTIOUS DISEASES AND DISEASES POTENTIALLY RESULTING FROM AN ACT OF BIOTERRORISM: REQUIRED HOSPITAL INFECTION CONTROL PRECAUTIONS[*]

Standard (handwashing)	Contact (gloves and gown[†])	Droplet (private room[‡] and surgical mask[§])	Airborne (private room,[‡] negative pressure room, HEPA filter mask)
All patients	MRSA, VRE	Meningococcal disease	Pulmonary TB
Anthrax	Enteric infections	Resistant pneumococci	Measles
Botulism	Skin infections	Pertussis	Varicella
Tularemia	Lice	Group A streptococci	Smallpox
Brucellosis	Scabies	Mycoplasma	
Q Fever	*Clostridium difficile* disease	Adenovirus	
Glanders	RSV, parainfluenza	Influenza	
Melioidosis	Certain VHFs	Pneumonic plague	
Ricin intoxication	• Ebola[¥]		
SEB intoxication	• Marburg[¥]		
T-2 intoxication	• Lassa Fever		
VEE, EEE, WEE	Smallpox		
	Melioidosis (with cutaneous lesions)		

[*]Thorough guidelines for hospital infection control can be found in: Cole LA. Bioterrorism threats: learning from inappropriate responses. *J Publ Hlth Manage Pract*. 2000;6:8–18.
[†]Gloves and/or gown should also be worn as a part of standard precautions (and other forms of precaution) when contact with blood, body fluids, and other contaminated substances is likely.
[‡]Mixing patients with the same disease is an acceptable alternative to a private room.
[§]Surgical masks should also be employed as a part of standard and contact precautions (along with eye protection and a face shield) if procedures are likely to generate splashes or sprays of infectious material.
[¥]While Ebola is transmitted primarily via infected blood and body fluids, the voluminous emesis and diarrhea produced by Ebola patients, the very low infectious inoculum of the virus, and the ease with which hemorrhagic respiratory secretions can be aerosolized during coughing, the CDC now recommends that both contact and droplet precautions be employed when managing Ebola victims; similar caution would likely apply to Marburg (and perhaps other VHF) patients as well.
EEE: eastern equine encephalomyelitis; HEPA: high-efficiency particulate air; MRSA: methicillin-resistant *Staphylococcus aureus*; RSV: respiratory syntactical virus; SEB: staphylococcal enterotoxin B; TB: tuberculosis; VEE: Venezuelan equine encephalitis; VHF: viral hemorrhagic fever; VRE: vancomycin-resistant enterococci; WEE: western equine encephalomyelitis

health departments, practitioners would access the state health department directly. Once alerted, local and regional health authorities know how to request additional support from health officials at higher jurisdictions. Each practitioner should have a point of contact with such agencies and should be familiar with mechanisms for contacting them before a crisis arises.

If an outbreak proves to be the result of terrorism, or if the scope of the outbreak overwhelms local resources, a regional or national response becomes imperative. Under such circumstances, an extensive panoply of supporting assets and capabilities may be summoned. The National Incident Management System and its component Incident Command System (ICS) provide a standardized approach to command and control at an incident scene.[49] Local officials use the ICS when responding to both natural and human-made disasters, and ICS would be equally applicable in responding to a biological attack. Under the ICS, a designated official, typically the fire chief or the chief of police, serves as local incident commander. The incident commander may be able to summon groups of volunteer medical personnel through the Metropolitan Medical Response System, which includes medical strike teams in 124 local jurisdictions. These teams, under contract with mayors of the 124 municipalities, are organized under the Department of Homeland Security's Office of Domestic Preparedness.

In any incident or disaster, whether natural or human-made, the local incident commander may request assistance from the state through the state coordinating officer if it appears that local resources or capabilities will be exceeded. The state coordinating officer works with the governor and other state of-

EXHIBIT 5-5

THE LABORATORY RESPONSE NETWORK

Sentinel laboratories. These laboratories, found in many hospitals and local public health facilities, have the ability to "rule-out" specific bioterrorism threat agents, to handle specimens safely, and to forward specimens on to higher echelon laboratories within the network.

Reference laboratories. These laboratories, typically found at state health departments, and at military, veterinary, agricultural, and water-testing facilities, can employ BSL-3 practices, and can often conduct nucleic acid amplification and molecular typing studies. The more than 100 reference laboratories can confirm ("rule-in") the presence of the various biological threat agents.

National laboratories. These laboratories, including those at the CDC and USAMRIID, can employ BSL-4 practices, and serve as the final authority in the work-up of bioterrorism specimens. These laboratories provide specialized reagents to lower level laboratories and have the ability to bank specimens, perform serotyping, and detect genetic recombinants and chimeras.

BSL: biosafety level
CDC: Centers for Disease Control and Prevention
USAMRIID: US Army Medical Research Institute of Infectious Disease

EXHIBIT 5-6

BIOSAFETY LEVELS

Biosafety Level 1: includes practices employed by a microbiology laboratory that deals only with well-characterized organisms that do not typically produce disease in humans. Work is conducted on open bench tops using standard microbiologic practices. Example: high school biology laboratory

Biosafety Level 2: includes practices employed by laboratories that deal with most human pathogens of moderate potential hazard. Laboratory coats and gloves are typically worn, access to the laboratory is restricted to trained personnel, and safety cabinets are often employed. Example: clinical hospital laboratory

Biosafety Level 3: Includes practices employed by laboratories that work with agents with the potential to cause serious and lethal disease by the inhalational route of exposure. Work is generally conducted in safety cabinets, workers are often vaccinated against the agents in question, and respiratory protection is worn. Clothing (such as scrub suits) is exchanged upon exiting the laboratory. Laboratories are negatively pressurized. Example: state health department laboratory

Biosafety Level 4: Also includes practices employed by laboratories working with highly hazardous human pathogens infectious via the inhalational route. BSL-4 organisms differ from those requiring BSL-3 precautions in that no vaccine or antibiotic therapy is available. Personnel may only enter the laboratory through a series of changing and shower rooms. Equipment and supplies enter via a double-door autoclave. Strict and sophisticated engineering controls are employed and personnel wear sealed positive-pressure space suits with supplied air. Laboratories are negatively pressurized. Examples: laboratories at the CDC, USAMRIID, the Canadian Science Center for Human and Animal Health, and a few other research facilities

BSL: biosafety level
CDC: Centers for Disease Control and Prevention
USAMRIID: US Army Medical Research Institute of Infectious Disease

ficials to make state-level assets (such as state health departments, state public health laboratories, and state police assets) available. Most state public health laboratories participate as "reference" laboratories in the Association of Public Health Laboratories and CDC's Laboratory Response Network. These facilities support hundreds of "sentinel" laboratories in local hospitals throughout the nation, and can provide sophisticated confirmatory diagnosis and typing of biological agents[50] (an overview of public health laboratory capa-

bilities is provided in Exhibit 5-5; the biosafety-level[51] precautions they employ are outlined in Exhibit 5-6). State police can provide law enforcement assistance and state police laboratories can assist with forensic analysis. Finally, governors can access military assets at the state level through National Guard units under their direct control. These units can provide law enforcement, public works assistance, mobile field hospital bed capacity, and other support. Every state governor now has, at his or her disposal, one of some 57 military Weapons of Mass Destruction–Civil Support Teams (WMD-CSTs). These 22-person advisory teams can offer expertise and provide liaison to additional military assets at the federal level.

When state capabilities are overwhelmed or insufficient, the state coordinating officer may alert the federal coordinating officer, who can, in turn, assist in activating the national response framework. The national response framework guides delivery of federal assets and provides for a coordinated multiagency federal response. Federal response and support to state and local jurisdictions, according to the framework, is organized into 15 emergency support functions (ESFs). ESF 8 provides for health and medical services. While a specific agency is assigned primary responsibility for each of the 15 ESFs, more than two dozen federal agencies, as well as the American Red Cross, can, under federal law, be tasked to provide assistance. Federal disaster medical support is primarily the responsibility of the Department of Health and Human Services which, through its Office of Emergency Response, oversees the National Disaster Medical System (NDMS).[52] A principal component of the NDMS is its network of disaster medical assistance teams, each of which consists of trained medical volunteers with the ability to arrive at a disaster site within 8 to 16 hours. Another important component of the NDMS is its excess hospital bed capacity, held at numerous Department of Veterans Affairs, military, and civilian hospitals throughout the nation.

Finally, several other federal agencies may play an important role in the response to disasters, including, in particular, those resulting from a biological attack. The CDC and USAMRIID provide national laboratories, which support the reference labs at the state level and are capable of dealing with virtually all potential biological threat agents.[53] Expert consultation and epidemiological investigative assistance is also available through the CDC, and bioweapons threat evaluation and medical consultation is likewise available through USAMRIID. Additionally, the military can provide expert advice and assistance to civilian authorities through Army National Guard's CBRNE Enhanced Response Force Package Teams, which can arrive at a disaster site within a few hours of notification, as well as through the aforementioned CBIRF, which is capable of providing reconnaissance, decontamination, and field treatment. Military support, when provided, would be subordinate to civilian authorities and would be provided and tailored by the Joint Task Force for Civil Support, a component of US Army Northern Command that provides a command-and-control element for all military assets involved in disaster response missions and other contingencies within the United States. Finally, the CDC has developed the Strategic National Stockpile, whereby critical drugs and vaccines necessary to combat a large disaster or terrorist attack are stockpiled at several locations throughout the country, available for rapid deployment to an affected area.[54] Release of stockpile components is currently controlled by the Department of Health and Human Services.

Step 9: Conduct an Epidemiological Investigation and Manage the Psychological Aftermath of a Biological Attack

Clinicians must be versed in the basic principles of epidemiology and be prepared to assist in the epidemiological investigation, which will be of paramount importance after a suspected terrorist attack. Although preventive medicine officers, environmental science officers, veterinarians, preventive medicine technicians (68S in US Army organizations), and field sanitation personnel may be invaluable in the course of such an investigation, the clinician should, nonetheless, have a working knowledge of the steps, known as the epidemiological sequence,[55] involved in the conduct of an epidemiological investigation

EXHIBIT 5-7

THE EPIDEMIOLOGICAL SEQUENCE

1. Make an observation
2. Count cases
3. Relate cases to population
4. Make comparisons
5. Develop the hypothesis
6. Test the hypothesis
7. Make scientific inferences
8. Conduct studies
9. Intervene and evaluate

Data source: Centers for Disease Control and Prevention. Investigating an outbreak. In: *Principles of Epidemiology: Self Study Course SS3030*. 2nd ed. Atlanta, GA: CDC; 1998: 347–424.

(Exhibit 5-7). Although the well-prepared clinician may positively impact the health and well-being of individual patients, it is only through the rapid conduct of a competent epidemiological investigation that large numbers of exposed persons are likely to be reached, and successful medical and psychological prophylaxis implemented, before the widespread outbreak of disease or panic.

In addition to the instigation of an epidemiological investigation and the institution of specific medical countermeasures against biological agent exposures, the clinician should be prepared to address the psychological effects of known, suspected, or feared exposure to threat agents.[56] An announced or threatened biological attack can provoke fear, uncertainty, and anxiety in the population, and can result in an overwhelming number of patients seeking evaluation and demanding therapy for feared exposure. Such a scenario might also follow the covert release of an agent once the resulting epidemic is characterized as being the consequence of a biological (or chemical or radiological) attack. Symptoms due to anxiety and autonomic arousal, as well as side effects from postexposure prophylactic drugs, may mimic prodromal disease due to biological agent exposure and pose dilemmas in differential diagnosis. Persons with symptoms arising from naturally occurring infectious diseases may likewise pose significant challenges to healthcare providers and public health officials.

Public panic and behavioral contagion are best prevented by timely, accurate, well-coordinated, and realistic risk communication from health and government authorities. Such communication should include an assessment of the risk of exposure, information regarding the resulting disease, and a recommended course of action for suspected exposure. As the epidemic subsides and public knowledge increases, public anxiety will decrease to realistic and manageable levels. This cycle of uncertainty, panic, response, and resolution occurred during the October 2001 anthrax bioterror event.[57] Readily accessible, biological, chemical, and radiological agent-specific information packages for local public health authorities and the general public are available through the CDC website, and can be of valuable assistance in risk communication.

Effective risk communication is possible only in the presence of well-conceived risk communication plans and tactics, worked out well in advance of an actual event. Similar advanced planning must take into account the need to rapidly establish local centers for the initial evaluation and administration of postexposure prophylaxis. Finally, the development of patient and contact tracing mechanisms and vaccine screening tools, the mechanisms for accession of stockpiled vaccines and medications, and the means by which to identify and prepare local facilities and healthcare teams for the care of mass casualties must be clearly elucidated in advance. The CDC's smallpox response plan[40] provides a useful template for such a coordinated, multifaceted approach, and the wisdom of farsighted planning and coordination was amply demonstrated by the efficient mass prophylaxis of over 10,000 individuals in New York City during the events surrounding the discovery of anthrax-contaminated mail in 2001.[58]

Step 10: Maintain a Level of Proficiency

Once response plans have been developed, they must be exercised. Military commanders and their units are typically well versed in planning and executing conventional field-training and command-post exercises. In the future, such exercises must account for the real possibility that military units may encounter biological weapons on the battlefield. Similarly, planning and exercises must account for the tandem threat posed by bioterrorist attacks against garrison activities. Local civilian exercises (which can often include military participants) are likewise a necessary component of disaster preparation. Such exercises should be designed so as to test incident command and control, communications, logistics, laboratory coordination, and clinical capabilities. These exercises may involve only the leadership of an organization and focus on planning and decision making (the command-post exercise), they may involve notional play around a tabletop exercise, or they may involve actual hands-on training and evaluation in a disaster drill or field-training exercise. In fact, the CDC expended considerable effort prior to the 2009 H1N1 influenza pandemic preparing for just such an event, conducting numerous tabletop and full-scale exercises involving CDC personnel as well as state public health participants. The Joint Commission requires hospitals to conduct a hazard vulnerability analysis, develop an emergency operations plan, and evaluate this plan twice yearly; one of these evaluations must include a community-wide drill.[59] Moreover, the Joint Commission specifically mandates that hospitals provide facilities (and training in the use of such facilities) for radioactive, biological, and chemical isolation and decontamination.

SUMMARY

Many resources, including this text, are now available to assist both military and civilian clinicians and public health professionals in planning for, and maintaining proficiency in, the management of real or threatened terror attacks. Finally, as discussed under step 8 above, numerous governmental, military, and civilian organizations have now been organized, trained, and equipped to provide assistance and consultation to clinicians, first responders, and public health officials faced with planning for and treating the victims of a potential terrorist attack. It is assistance that, if incorporated into thorough planning efforts, will hopefully never be needed for actual patient care purposes.

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Chapter 6

ANTHRAX

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- **Side Effects**

SUMMARY

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INTRODUCTION AND HISTORY

Anthrax, a zoonotic disease caused by *Bacillus anthracis*, occurs in domesticated and wild animals, primarily herbivores, including goats, sheep, cattle, horses, and swine.[1–5] Humans usually become infected by contact with infected animals or contaminated animal products, most commonly via the cutaneous route and only rarely via the respiratory or gastrointestinal routes.[6,7] Anthrax has a long association with human history. The fifth and sixth plagues described in Exodus may have been anthrax in domesticated animals followed by cutaneous anthrax in humans. Virgil described anthrax in domestic and wild animals in his *Georgics*, and anthrax was an economically important agricultural disease during the 16th through 18th centuries in Europe.[8,9]

Anthrax, which is intimately associated with the origins of microbiology and immunology, was the first disease for which a microbial origin was definitively established. Robert Koch established the microbial origin for anthrax in 1876.[10,11] Anthrax also was the first disease for which an effective live bacterial vaccine was developed; Louis Pasteur developed that vaccine in 1881.[12] Additionally, anthrax represents the first described occupational respiratory infectious disease. During the latter half of the 19th century, inhalational anthrax,[13] a previously unrecognized form, occurred among wool-sorters in England as a result of the generation of infectious aerosols of anthrax spores under industrial conditions from the processing of contaminated goat hair and alpaca wool.[14]

The military has long been concerned about *B anthracis* as a potential biological weapon because anthrax spores are infectious by the aerosol route, and a high mortality rate is associated with untreated inhalational anthrax. In 1979 the largest inhalational anthrax epidemic of the 20th century occurred in Sverdlovsk, Russia. *B anthracis* spores were accidentally released from a military research facility located upwind from where the cases occurred. According to the accounts provided by two Soviet physicians, 96 human anthrax cases were reported, of which 79 were gastrointestinal and 17 cutaneous. The 79 gastrointestinal cases resulted in 64 deaths.[15] Although the initial report of this event attributed the infections to a gastrointestinal source, later evidence indicated that an aerosol release of weaponized anthrax spores from a military production facility had occurred, and thus, inhalational anthrax was the predominant cause of these civilian casualties. Retrospective analysis using administrative name lists of compensated families, household interviews, grave markers, pathologists' notes, various hospital lists, and clinical case histories of five survivors yielded evidence of 77 anthrax cases, with 66 deaths and 11 survivors.[15] Cases were also reported in animals located more than 50 km from the site.[16,17] Polymerase chain reaction examination of tissue samples collected from 11 of the victims demonstrated that virulent *B anthracis* DNA was present in all these patients, and at least five different strains of virulent *B anthracis* were detected based on variable number tandem repeat analysis.[18]

The retrospective data associated with the Sverdlovsk incident as well as studies performed for the Department of Homeland Security have been used by several computer modeling efforts to better understand the human infectious dose.[19,20] Under the direction of the Department of Health and Human Services, the Office of the Assistant Secretary of Preparedness and Response, Public Health Emergency Medical Countermeasures Enterprise, and Biomedical Advanced Research and Development, these agencies have developed a variety of computer dissemination models for a wide variety of potential scenarios.

Although the Sverdlovsk incident is not well known among US civilians, most people are familiar with the 2001 bioterrorist attack in the United States in letters containing dried *B anthracis* spores. The spore powder, which was sealed in letters addressed to members of Congress and the press, was mailed through the US Postal Service.[21–24] According to the Centers for Disease Control and Prevention, 22 people contracted anthrax from the letters.[21,25–29] Of the 11 individuals who developed inhalational anthrax, five died and six survived after intensive antimicrobial therapy. Eleven other people contracted cutaneous anthrax; all survived after treatment. Thousands of other persons received prophylaxis with antibiotics and, in some cases, postexposure vaccination.[30–33]

Considerable research has been devoted to biodefense research and modeling since this event.[34–45] It has been estimated that the 2001 anthrax attacks cost the United States more than $1 billion in medical planning, response, and remediation costs.[46–49] Additionally, this incident profoundly affected the law enforcement, scientific, and medical communities within the United States and throughout the world. Although the source of these letters has never been definitively identified, the impact on biodefense research establishments has been a transformational event for researchers and institutes.

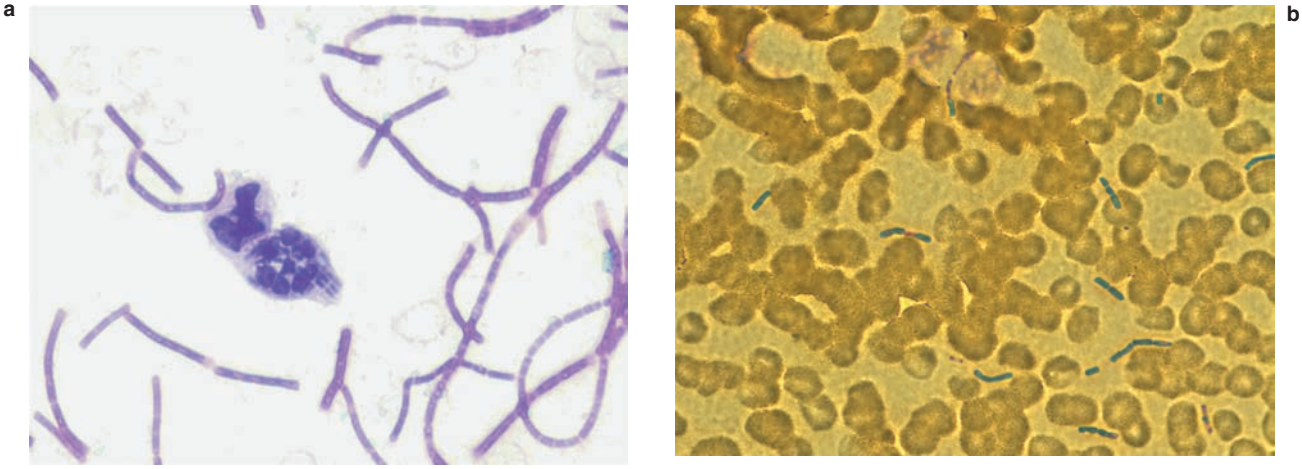


Figure 6-1. (**a**) Gram stain of a blood smear from an infected guinea pig demonstrating intracellular bacilli chains within a polymorphonuclear leukocyte. (**b**) Gram stain of peripheral blood smear from a nonhuman primate infected with *Bacillus anthracis*, Ames strain.
Photographs: (a) Courtesy of Susan Welkos, PhD, Division of Bacteriology, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland. (b) Courtesy of John Ezzell, PhD, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

THE ORGANISM

B anthracis is a large, gram-positive, spore-forming, nonmotile bacillus (1–1.5 μm × 3–10 μm) that is closely related to *Bacillus cereus* and *Bacillus thuringiensis*. The organism grows readily on sheep blood agar aerobically and is nonhemolytic under these conditions. The colonies are large, rough, and grayish white, with irregular, curving outgrowths from the margin. The organism forms a prominent capsule both in vitro in the presence of bicarbonate in the culture media and elevated levels of carbon dioxide in the bacterial plate incubator and in tissue in vivo. In tissue, the encapsulated bacteria occur singly or in chains of two or three bacilli (Figure 6-1). The organism does not form spores in living tissue; sporulation occurs only after the infected carcass tissues are exposed to oxygen. The spores, which cause no swelling of the bacilli, are oval and they occur centrally or paracentrally (Figure 6-2). *B anthracis* spores are composed of dozens of spore coat proteins that—in part—protect the genomic material housed in the core.[50,51] The spores are surrounded by a loose fitting membrane referred to as the exosporium. The exosporium has been shown to impact how the spore interacts with certain types of mammalian cells.[52,53] The spores, which are resistant to environmental stressors, may survive for decades in certain soil conditions. Bacterial identification is confirmed by demonstration of the protective antigen (PA) toxin component, lysis by a specific bacteriophage, detection of capsule by fluorescent antibody, and virulence for mice and guinea pigs.[54,55] Additional confirmatory tests to identify toxin and capsule genes by polymerase chain reaction, developed as research tools, have been

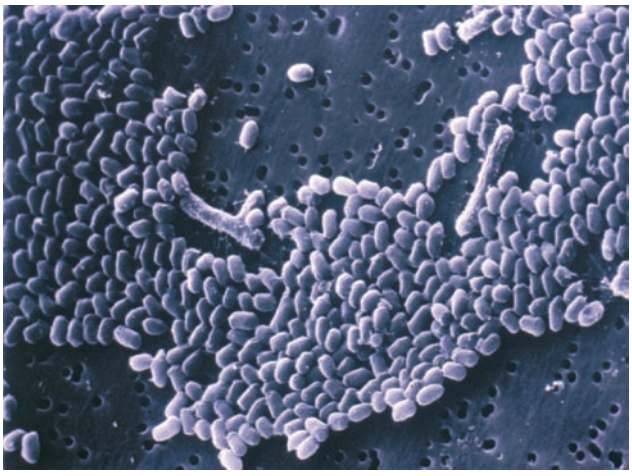


Figure 6-2. Scanning electron micrograph of a preparation of *Bacillus anthracis* spores. Two elongated bacilli are also presented among the oval-shaped spores. Original magnification × 2,620.
Photograph: Courtesy of John Ezzell, PhD, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

incorporated into the Laboratory Response Network established by the Centers for Disease Control and Prevention.[56–59]

The diagnosis of anthrax has been complicated by the identification of strains of *B cereus*, which produce anthrax-like disease. Because *B cereus* is hemolytic and resistant to the anthrax-specific gamma bacteriophage, such isolates would not typically be tested for the presence of genes encoding anthrax toxin, especially because *B cereus* is often regarded as an environmental contaminant.[60] Continued reports of bacterial strains harboring anthrax toxin genes have demonstrated not only the importance of appropriate detection strategies, but also the possibility of emerging risks associated with the possible transfer of *B anthracis* characteristics to other organisms.[61,62]

EPIDEMIOLOGY

B anthracis, an organism that exists in the soil as a spore, occurs worldwide. Whether its persistence in the soil results from significant multiplication of the organism, or from cycles of bacterial amplification in infected animals whose carcasses then contaminate the soil, remains unsettled.[63–67] The form of the organism in infected animals is the bacillus.

Domestic or wild animals become infected when they ingest spores while grazing on contaminated land or eating contaminated feed. Pasteur originally reported that environmental conditions such as drought, which may promote trauma in the oral cavity on grazing, may increase the chances of acquiring anthrax.[68] Spread from animal to animal by mechanical means—by biting flies and from one environmental site to another by nonbiting flies and by vultures—has been suggested to occur.[64,69]

Anthrax in humans is associated with agricultural, horticultural, or industrial exposure to infected animals or contaminated animal products. In less developed countries, primarily Africa, Asia, and the Middle East, disease occurs from contact with infected domesticated animals or contaminated animal products. Contact may include handling contaminated carcasses, hides, wool, hair, and bones or ingesting contaminated meat. Cases associated with industrial exposure—rarely seen—occur in workers processing contaminated hair, wool, hides, and bones. Direct contact with contaminated material leads to cutaneous disease, and ingestion of infected meat leads to oropharyngeal or gastrointestinal forms of anthrax. It has been well documented that intravenous drug users can become infected with *B anthracis*, resulting in a septicemic form of anthrax.[70–78] Inhalation of a sufficient quantity of spores, usually seen only during generation of aerosols in an enclosed space associated with processing contaminated wool or hair, leads to inhalational anthrax. Military research facilities have played a major role in studying and defining anthrax, as well as many other zoonotic diseases in wild and domestic animals and the subsequent infections in humans.[79]

Unreliable reporting makes it difficult to estimate with accuracy the true incidence of human anthrax. It was estimated in 1958 that between 20,000 and 100,000 cases occurred annually worldwide.[80] In more recent years, anthrax in animals has been reported in 82 countries, and human cases continue to be reported from Africa, Asia, Europe, and the Americas.[81–85] In the 1996–1997 global anthrax report, a general decrease appeared in anthrax cases worldwide; however, anthrax remains underdiagnosed and underreported.[86]

In the United States the annual incidence of human anthrax has steadily declined from about 127 cases in the early part of the 20th century to about 1 per year for the past 10 years.[87] The vast majority of these cases have been cutaneous. Under natural conditions, inhalational anthrax is rare; before the anthrax bioterrorism event in 2001, only 18 cases had been reported in the United States in the 20th century.[88,89] In the early part of the 20th century, inhalational anthrax cases were reported in rural villagers in Russia who worked with contaminated sheep wool inside their homes.[90] However, in recent years a significant decrease occurred in anthrax cases in domestic animals in east Russia. Five inhalational anthrax cases occurred in woolen mill workers in New Hampshire in the 1950s.[91] During economic hardship and disruption of veterinary and human public health practices (eg, during wartime), large anthrax epidemics have occurred. The largest reported human anthrax epidemic occurred in Zimbabwe from 1978 through 1980, with an estimated 10,000 cases.[92]

Essentially all cases were cutaneous, including rare gastrointestinal disease cases and eight inhalational anthrax cases, although no autopsy confirmation was reported.[93]

PATHOGENESIS

B anthracis produces two protein exotoxins, known as the lethal toxin (LT) and the edema toxin (ET); an antiphagocytic capsule; and other known and putative virulence factors.[94] The role of the capsule in pathogenesis was demonstrated in the early 1900s, when anthrax strains lacking a capsule were shown

to be attenuated.[95] In more recent years, the genes encoding synthesis of the capsule were identified on the 96-kilobase plasmid known as pXO2. Molecular analysis revealed that strains cured of this plasmid no longer produced the capsule and were attenuated, thus confirming the critical role of the capsule in virulence.[96] The capsule is composed of a polymer of D-glutamic acid, which confers resistance to phagocytosis and may contribute to the resistance of anthrax to lysis by serum cationic proteins.[97–102] Capsule production is necessary for dissemination to the spleen in a murine inhalational anthrax model.[103] The capsule has also been the focus of several efforts to develop new generation anthrax vaccines.[104–106] Evidence indicates that the capsule may enhance the protection afforded by PA-based vaccines against anthrax if opsonizing antibodies are produced.[106]

Koch first suggested the importance of toxins in his initial studies on anthrax. In 1954 Smith and Keppie[107] demonstrated a toxic factor in the serum of infected animals that was lethal when injected into other animals. The role of toxins in virulence and immunity was firmly established by many researchers in the ensuing years.[108,109] Advances in molecular biology have produced a more complete understanding of the biochemical mechanisms of action of the toxins, and they have begun to provide a more definitive picture of their role in the pathogenesis of the disease.

Two protein exotoxins, known as the LT and the ET, are encoded on a 182-kb plasmid (pXO1), distinct from that coding for the capsule. In an environment of increased bicarbonate in the growth media, atmospheric carbon dioxide within the plate incubation chamber, and increased temperature, such as is found in the infected host, transcription of the genes encoding these and other virulence-associated gene products is enhanced.[94,110–113] A complex regulatory cascade controlled in large part by the *atxA* and *acpA* genes encoded on the toxin plasmid pXO1 and pXO2, respectively, directs the production of virulence factors in response to these environmental signals.[114,115] The anthrax toxins, like many bacterial and plant toxins, possess two components: (1) a cell binding, pore-forming, or B, domain; and (2) an active, or A, domain that has the toxic and—usually—the enzymatic activity (Figure 6-3). The B and A anthrax toxin components, which are synthesized from different genes, are secreted as noncovalently linked proteins. The anthrax toxins are unusual because both toxins share the B protein, PA. Thus, the LT is composed of the PA63 (MW [molecular weight] 63,000 after cleavage from a MW 83,000 protein) heptamer or octamer combined with a second protein, which is known as lethal factor (LF [MW 90,000]), and the ET is composed of PA complexed with the edema factor (EF [MW 89,000]). Each of these three toxin proteins—the B protein and both A proteins—individually is without biological activity. The critical role of the toxins in pathogenesis was established when it was shown that deletion of the toxin-encoding plasmid pXO1[96,116] or the PA gene alone[117] attenuates the organism. Crude toxin preparations have been shown to impair neutrophil chemotaxis[118,119] and phagocytosis.[97]

The ET, which causes edema when injected into the skin of experimental animals, is likely responsible for the marked edema often present at bacterial replication sites.[120,121] This toxin is a calmodulin-dependent adenylate cyclase that impairs phagocytosis and priming for the respiratory burst in neutrophils; it also inhibits the production of interleukin-6 and tumor necrosis factor by monocytes, which may further weaken host resistance.[122–124] ET also impairs dendritic cell function and appears to act with LT to suppress the innate immune response.[125]

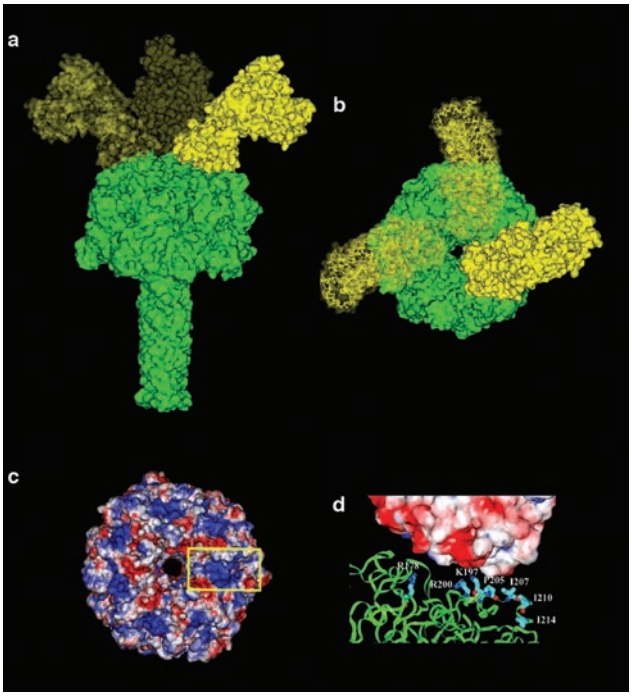


Figure 6-3. Composition of anthrax lethal protein toxin. Molecular models of the protective antigen $(PA)_{63}$ heptamer and the PA_{63} heptamer-lethal factor (LF) complex. (**a, b**) Side and top views of PA_{63} heptamer (*green*) bound to three LF molecules (*yellow*). (**c, d**) The surface renderings are colored according to the negative (*red*) and positive (*blue*) electrostatic surface potential. (**c**) Top view of the PA_{63} heptamer. The *yellow box* highlights the protomer-protomer interface and where LF binds to heptameric PA. (**d**) A hypothetical PA_{63} heptamer–LF interface.
Photographs: Courtesy of Kelly Halverson, PhD, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

The LT is a zinc metalloprotease that is lethal for experimental animals[120,121,126] and is directly cytolytic for rodent macrophages, causing release of the potentially toxic cytokines interleukin-1 and tumor necrosis factor.[127] In in vitro models, LT cleaves members of the mitogen-activated protein kinase (MAPK) kinase family, which are an integral part of a phosphorelay system that links surface receptors to transcription of specific genes within the nucleus. Thus, LT interferes with the MAPK signaling pathways necessary for many normal cell functions.[128] In macrophage and dendritic cell models, LT leads to inhibition of proinflammatory cytokines, downregulation of costimulatory molecules, and ineffective T-cell priming.[128–131] In vitro it also appears to promote apoptosis of endothelial cells lining the vascular system, leading to speculation that LT-induced barrier dysfunction leads to the vascular permeability changes accompanying systemic anthrax infection.[132] Effects on hormone receptors, including glucocorticoids, have also been reported. Although much of the information regarding LT activity has been obtained from animal-derived cell culture models, Fang et al reported that—in vitro—LT inhibits MAPK kinase dependent interleukin-2 production and proliferative responses in human CD4+ T cells.[133] Studies using tissue-specific CMG2 knockout mice strongly indicate that LTs/ETs target myeloid-derived cells to promote bacterial survival early in infection.[134] In addition, the data suggest that elevated levels of toxin specifically target host organs and are responsible for the significant morbidity and mortality caused by anthrax infection.[135]

Studies in cell culture models have provided a clearer understanding of the molecular interactions of the toxin proteins.[128] PA first binds, most likely by a domain at its carboxy-terminus, to a specific cell receptor.[136–138] Two proteins have been proposed as the PA receptor: (1) Tumor endothelial marker 8 TEM8, (ANTX1); and (2) capillary morphogenesis protein, CMG2 (ANTX2).[139–141] Both receptors have a von Willibrand factor type A domain that appears to interact with PA. Once bound, PA is cleaved by a furin-like protease, resulting in retention of a 63-kilodalton fragment of PA on the cell surface.[142,143] This cleavage promotes formation of PA heptamers and creates a binding site on PA to which up to three molecules of the LF and the EF can bind with high affinity.[129] Heptamerization[144] and octamerization[141–144] stimulates endocytosis of PA (or PA EF or PA LF complexes), which are then delivered into early endosomes. The mildly acidic pH of the endosome is hypothesized to trigger membrane insertion of the heptameric PA into intraluminal vesicles.[145] EF and LF are translocated into the lumen of the vesicle and are thereby protected from lysosomal proteases.[145] The toxins are then translocated via endosomal carrier vesicles to the cell cytosol, where they express their toxic activity.[145] In addition, studies have also suggested that the formation of octamers provides stability to these toxin products and permits active LT to travel freely in the circulatory system.[146]

The processes leading to toxin activity in the infected animal may be more complicated because the toxin proteins appear to exist in the serum as a complex of PA and EF/LF.[147] The proteolytic activation of PA necessary to form LT or ET may occur in interstitial fluid or serum rather than on the cell surface.[147] The LT or ET may then bind to target cells and be internalized. This theory was bolstered by Panchal et al who demonstrated that purified LF complexed with the PA heptamer cleaved both a synthetic peptide substrate and endogenous MAPK kinase substrates and killed susceptible macrophage cells.[148] In addition, complexes of the heptameric PA-LF found in the plasma of infected animals showed functional activity.[148] Terminally, toxin is present in very high concentrations in the blood, which probably accounts for the sudden death observations in infected experimental animals.

Although these toxins were once thought to be exclusively found in *B anthracis*, recent cases of inhalational disease have been identified that possess the hallmarks of anthrax disease; however, the bacteria recovered were not *B anthracis* but did possess anthrax toxin genes.[149–152] Studies have identified isolates of *B cereus* that carried a plasmid homologous to the anthrax toxin plasmid pXO1. The polyglutamate capsule was not produced by this *B cereus* isolate. However, gene sequences encoding a polysaccharide capsule were present on a smaller plasmid.[149] Capsule-producing strains of *B cereus* have caused severe pneumonia.[150] Consequently, a possibility of false positives exists in diagnostic tests that rely on toxin-based identification of genes or gene products. Subsequent investigations of these strains determined that the virulence of these strains in mice, guinea pigs, and rabbits was significantly attenuated when compared to fully virulent *B anthracis*.[153,154] It was also shown that vaccines that are effective against fully virulent *B anthracis* can protect mice and guinea pigs from infection with the anthrax-like *B cereus* strain.[154]

Infection begins when the spores are introduced through the skin or mucosa. Spores are then ingested at the local site by macrophages. Phagocytosed spores can have multiple fates depending on the stage of infection and the spore burden of individual phagocytes.[155,156] Within the lungs, spores are translocated by pulmonary macrophages and dendritic cells. Phagocytes have a dual role; they can transport spores to the lymphatic system[157–160] but also are bactericidal toward

germinating spores under certain conditions.[158,161–163] Another hypothesis has been proposed that may explain the toxins' effects early in the infectious process. Banks, Ward, and Bradley[164] have hypothesized that intoxication may occur after spores have been engulfed by phagocytic cells. The anthrax toxin receptors have been located on the inside of the phagolysosome, and the germinating spore may secrete toxins that interact with these receptors within the phagolysosome. The effector molecules (EF and/or LF) can then be translocated into the cytoplasm.[155,164]

Once a spore becomes vegetative, it can produce a robust capsule and large amounts of toxins. At these sites, the bacteria proliferate and produce the ETs and LTs that impair host leukocyte function and lead to the distinctive pathological findings: edema, hemorrhage, tissue necrosis, and a relative lack of leukocytes. Once the vegetative cells emerge from the phagolysosome, they replicate within the cell and finally exit through the host cell plasma membrane.[160] In inhalational anthrax, the spores are ingested by alveolar phagocytes, which transport them to the regional tracheobronchial lymph nodes, where germination occurs.[165]

Anthrolysin O (ALO) and phospholipases may also play critical roles as virulence factors for *B anthracis*[166] and mediate the toxicity of *B anthracis* to lung epithelial cells under microaerobic conditions.[167] ALO has been found to cause lysis of human phagocytes and epithelial cells. The mechanism of action appears to be from ALO pore-forming alterations of the cellular membrane, resulting in acute primary membrane permeabilization followed by a burst of reactive radicals released from the mitochondria.

The evidence reported from animal studies overwhelmingly suggests that the alveolar spaces are not permissive for significant levels of spore germination. Rather, spores begin to germinate once phagocytosed during translocation to and upon deposition within lymph nodes.[165,168–171] However, several studies have suggested that small amounts of germination may occur within the alveolar spaces.[171,172] Additionally, the nasal-associated lymphoid tissue has been explored as another area from which infection may be initiated.[171,173,174] These data, largely collected through in-vivo imaging technologies, suggest that other scenarios may lead to spore germination after inhalation.[159] Once in the tracheobronchial lymph nodes, the local production of toxins by extracellular bacilli generates the characteristic pathology picture: massive hemorrhagic, edematous, and necrotizing lymphadenitis; and mediastinitis (the latter is almost pathognomonic of this disease).[175]

These findings in human disease have been replicated in various animal disease models.[176,177] The bacilli can then spread to the blood, leading to septicemia with seeding of other organs and frequently causing hemorrhagic meningitis. Death is most likely the result of systemic inflammatory response syndrome triggered by the release of endogenous cellular contents from damaged or dying cells, termed damage-associated molecular patterns and in combination with exogenous microbial exposure or pathogen-associated molecular patterns,[178] resulting in respiratory failure associated with pulmonary edema, direct cardiac tissue damage, overwhelming bacteremia, accompanied frequently with meningitis.

CLINICAL DISEASE

The military seeks to defend against anthrax used as an inhalational biological weapon. However, other anthrax forms are more likely to be seen by medical officers—particularly when deployed to third world countries—and are therefore included for completeness.

Cutaneous Anthrax

More than 95% of anthrax cases are cutaneous.[179–181] After inoculation, the incubation period is 1 to 5 days. The disease first appears as a small papule that progresses over a day or two to a vesicle containing serosanguineous fluid with many organisms and a paucity of leukocytes. Histopathology findings consist of varying degrees of ulceration, vasculitis, perivascular inflammation, coagulative necrosis, hemorrhage, and edema.[182] The vesicle—which may be 1 to 2 cm in diameter—ruptures, leaving a necrotic ulcer (Figure 6-4). Satellite vesicles may also be present. The lesion is usually painless, and varying degrees of edema may be present around it.[183] The edema may occasionally be massive, encompassing the entire face or limb, which is described as "malignant edema." Patients usually have fever, malaise, and headache, which may be severe in those with extensive edema. There may also be local lymphadenitis. The ulcer base develops a characteristic black eschar, and after 2 to 3 weeks the eschar separates, often leaving a scar and sometimes requiring surgical reconstruction.[184,185] Debridement has been shown to improve survival rates in a mouse model of subcutaneous anthrax[159]; however, no clinical studies have been conducted to validate this procedure in human clinical disease. Septicemia is rare, and with treatment, mortality should be less than 1%.[184,186–188] In addition, no age-related risk factor appears to be associated with cutaneous human anthrax.[189]



Figure 6-4. Cutaneous lesions of anthrax. (**a**) Ulcer with vesicle ring. (**b**) Black eschar with surrounding erythema. (**c**) Marked edema of extremity secondary to anthrax edema toxin with multiple black eschar.
Photographs: Courtesy of the Centers for Disease Control and Prevention, Atlanta, Georgia. www.bt.cdc.gov/agent/anthrax/anthrax-images/cutaneous.asp.

Of recent interest has been the identification of anthrax cases among intravenous drug users in western Europe.[70–75] In 2000 a case of cutaneous anthrax was identified in a Norwegian patient who participated in subdermal drug injection, commonly known as "skin popper."[76] The first reported case of intravenous drug user-associated anthrax was in Scotland with subsequent 47 confirmed cases and 13 fatalities. These numbers increased to a total of 119 cases from December 2009 to December 2010.[74] This disease is thought to be initiated by direct injection of spore-contaminated heroin, which led to clinical presentations ranging from subcutaneous disease to septicemic anthrax.[70–78,190–192]

Inhalational Anthrax

Inhalational anthrax begins after an incubation period of 1 to 6 days with nonspecific symptoms of malaise, fatigue, myalgia, and fever.[193–195] A nonproductive cough and mild chest discomfort may also occur. These symptoms usually persist for 2 or 3 days, and in some cases there may be a short period of improvement. Then a sudden onset of increasing respiratory distress with dyspnea, stridor, cyanosis, increased chest pain, and diaphoresis occurs. Associated edema of the chest and neck may also be present. Chest radiograph examination usually shows the characteristic widening of the mediastinum from necrosis and hemorrhage of the lymph nodes and surrounding tissues, often with associated pleural effusions (Figure 6-5). In the 2001 bioterrorist event, the pleural effusions were initially small but rapidly progressed and persisted despite effective antibiotic therapy.[195,196] The effusions were predominantly serosanguineous, and immunohistochemistry revealed the presence of *B anthracis* cell wall and capsule antigens. Effusion fluid from deceased patients who had received fewer than 55 hours of antibiotic therapy revealed bacilli.[197]

Polymerase chain reaction analysis of the pleural fluid was also positive for *B anthracis* DNA.[198] Pneumonia has not been a consistent finding but can occur in some patients and may be attributed to vascular permeability, intra-alveolar edema, and hyaline membrane formation.[197] Although inhalational anthrax cases have been rare in this century, except for the 11 cases arising from the anthrax letters in 2001, several cases have occurred in patients with underlying pulmonary disease, suggesting that this condition may increase susceptibility to the disease.[68] Meningitis is present in up to 50% of cases, and some patients may present with seizures. The onset of respiratory distress is fol-

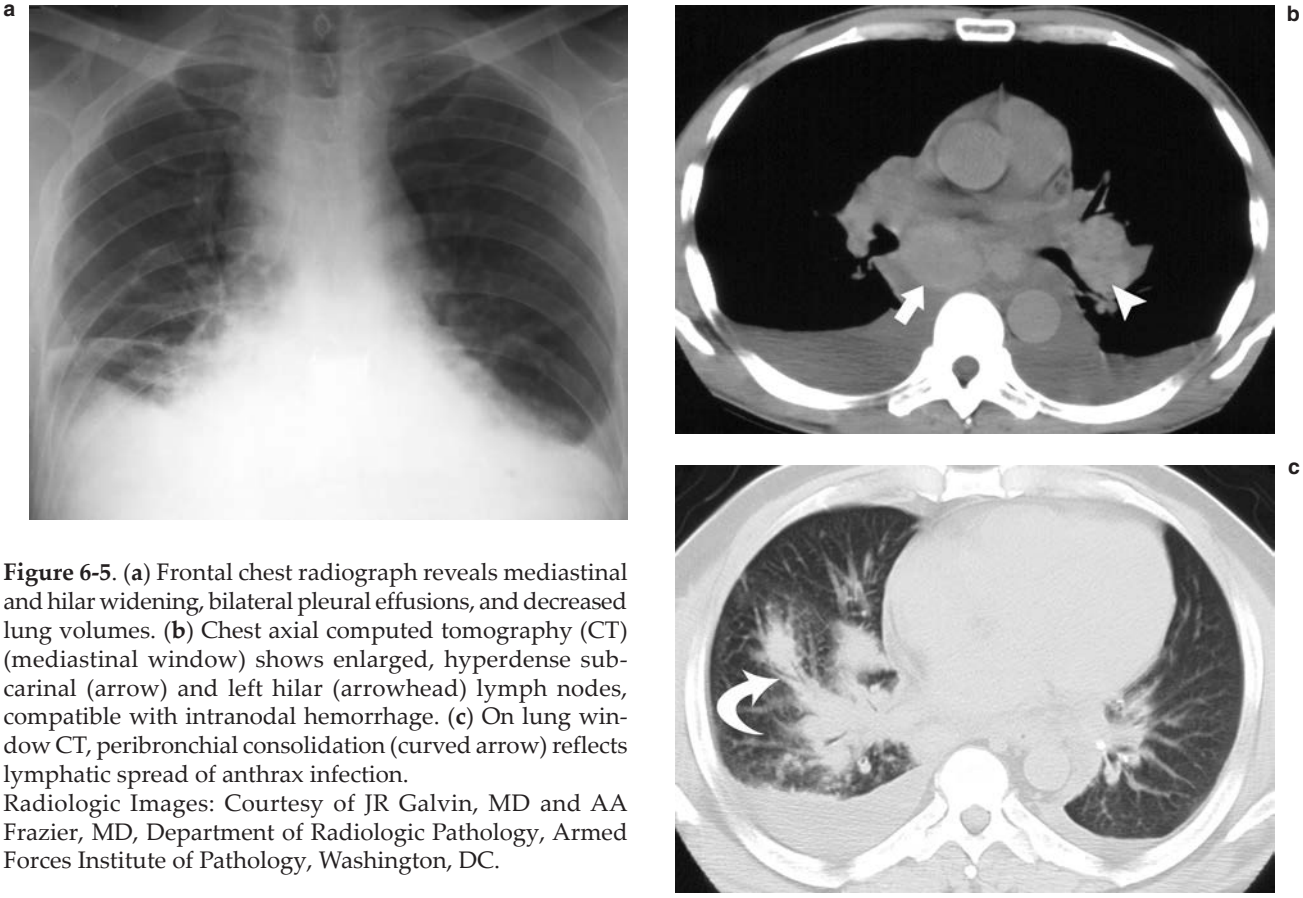


Figure 6-5. (**a**) Frontal chest radiograph reveals mediastinal and hilar widening, bilateral pleural effusions, and decreased lung volumes. (**b**) Chest axial computed tomography (CT) (mediastinal window) shows enlarged, hyperdense subcarinal (arrow) and left hilar (arrowhead) lymph nodes, compatible with intranodal hemorrhage. (**c**) On lung window CT, peribronchial consolidation (curved arrow) reflects lymphatic spread of anthrax infection.
Radiologic Images: Courtesy of JR Galvin, MD and AA Frazier, MD, Department of Radiologic Pathology, Armed Forces Institute of Pathology, Washington, DC.

lowed by the rapid onset of shock and death within 24 to 36 hours. Mortality had been essentially 100% in the absence of appropriate treatment; however, during 2001 the mortality rate was 45%.[195,196]

An inhalational pulmonary disease thought initially to be anthrax has been identified to be caused by *B cereus* strains.[152,199] These cases were found in metal welders, and susceptibility of these patients to this unusual pathogen may be related to inhalation of heavy metals during welding. Heavy metal exposure produces immunosuppression and an increased susceptibility to infection.

Meningitis

Meningitis may occur after bacteremia as a complication of any of the disease's clinical forms.[190–192] Meningitis may also occur—rarely—without a clinically apparent primary focus, and it is often hemorrhagic, which is important diagnostically, and almost always fatal (Figure 6-6). Studies have suggested that LF, EF, and protease InhA inhibit neutrophil signaling pathways in brain endothelium, thus promoting anthrax meningitis.[193–195]

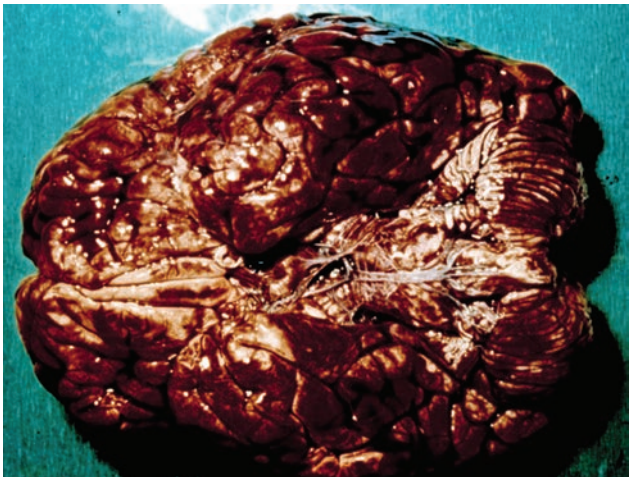


Figure 6-6. Meningitis with subarachnoid hemorrhage in a man from Thailand who died 5 days after eating undercooked carabao (water buffalo).
Reproduced from: Binford CH, Connor DH, eds. *Pathology of Tropical and Extraordinary Diseases.* Vol 1. Washington, DC: Armed Forces Institute of Pathology; 1976: 121. AFIP Negative 75-12374-3.

Oropharyngeal and Gastrointestinal Anthrax

Oropharyngeal and gastrointestinal anthrax result from ingesting infected meat that has not been sufficiently cooked or by ingesting anthrax spores either directly or from swallowing contaminated respiratory secretions.[178,200,201] After an incubation period of 2 to 5 days, patients with oropharyngeal disease present with severe sore throat or a local oral or tonsillar ulcer, usually associated with fever, toxicity, and swelling of the neck resulting from cervical or submandibular lymphadenitis and edema. Dysphagia and respiratory distress may also be present. Gastrointestinal anthrax begins with nonspecific symptoms of nausea, vomiting, and fever; in most cases severe abdominal pain follows. The presenting sign may be an acute abdomen, which may be associated with hematemesis, massive ascites, and bloody diarrhea. Mortality in both forms may be as high as 50%, especially in the gastrointestinal form.

DIAGNOSIS

The most critical aspect in making an anthrax diagnosis is a high index of suspicion associated with a compatible history of exposure. Cutaneous anthrax should be considered after a painless pruritic papule, vesicle, or ulcer develops—often with surrounding edema—and then becomes a black eschar. With extensive or massive edema, such a lesion is almost pathognomonic. Gram stain or culture of the lesion usually confirms the diagnosis. Bacterial culture tests include colony morphology on sheep blood agar plates incubated at 35°C to 37°C for 15 to 24 hours. *B anthracis* colonies are 2 to 5 mm in diameter, flat or slightly convex, irregularly round with possible comma-shaped ("Medusa-head") projections with a ground-glass appearance (Figure 6-7). The colonies tend to have tenacious consistency when moved with a bacterial loop and are not β-hemolytic. The bacteria appear as gram-positive, 1 to 8 μm long and 1 to 1.5 μm wide bacilli. India ink staining reveals capsulated bacteria. A motility test should be performed either by wet mount or motility media; *B anthracis* is nonmotile. Gamma bacteriophage lysis and direct fluorescent antibody tests are performed at Level D laboratories as confirmatory tests (Figures 6-7 and 6-8). Commercial polymerase chain reaction kits specific for the *B anthracis* pXO1 and pXO2 plasmids are also available to assist in identifying this organism. The differential diagnosis should include tularemia, staphylococcal or streptococcal disease, and orf (a viral disease of sheep and goats transmissible to humans).

The diagnosis of inhalational anthrax is difficult, but the disease should be suspected with a history of exposure to a *B anthracis*–containing aerosol. The early

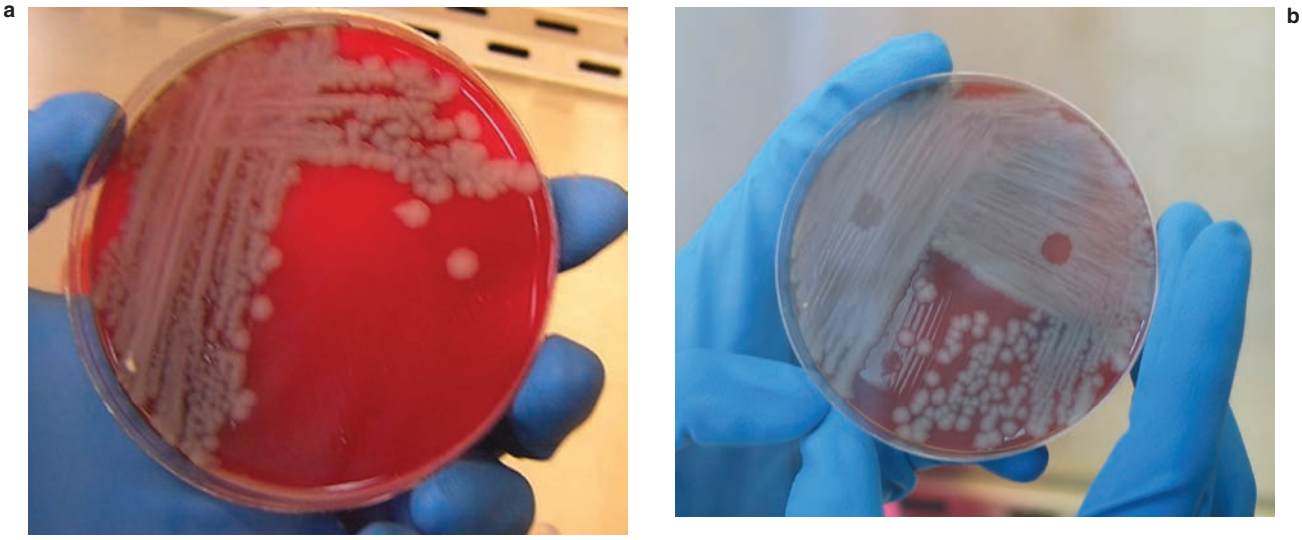


Figure 6-7. (**a**) Isolated colonies of *Bacillus anthracis* on sheep blood agar plate. (**b**) Detection of *B anthracis* using specific gamma-phage mediated cell-lysis.
Photographs: Courtesy of Bret K Purcell, PhD, MD, Division of Bacteriology, US Army Medical Research Institute of Infectious Diseases and the Defense Threat Reduction Agency/Threat Agent Detection and Response Program, National Center for Disease Control, Tbilisi, Georgia, 2005.

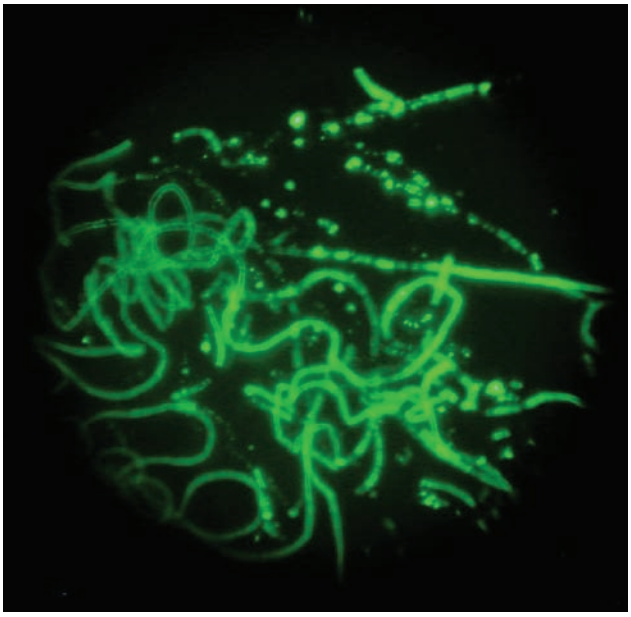


Figure 6-8. Direct fluorescent antibody stain of *Bacillus anthracis* capsule.
Photograph: Courtesy of David Heath, PhD, Division of Bacteriology, US Army Medical Research Institute of Infectious Diseases and the Defense Threat Reduction Agency/Threat Agent Detection and Response Program, National Center for Disease Control, Tbilisi, Georgia, 2005.

symptoms are nonspecific[194,202–204] and include fever, chills, dyspnea, cough, headache, vomiting, weakness, myalgias, abdominal pain, and chest or pleuritic pain. This stage of the disease may last from hours to a few days. However, the development of respiratory distress in association with radiographic evidence of a widened mediastinum resulting from hemorrhagic mediastinitis and the presence of hemorrhagic pleural effusion or hemorrhagic meningitis should suggest the diagnosis. Contrast-enhanced computer tomography images reveal diffuse hemorrhagic mediastinal and hilar adenopathy with edema, perihilar infiltrates, bronchial mucosal thickening, and hemorrhagic pleural, and pericardial effusions.[205] During the later stages of the disease patients develop sudden fever, dyspnea, diaphoresis, cyanosis, hypotension, shock, and death.[202] Blood culture should demonstrate growth in 6 to 24 hours if the patient has not received antibiotics before collection, and a Gram stain of peripheral blood smears often reveals large bacilli in later disease stages. Sputum examination is not helpful in making the diagnosis because pneumonia is usually not a feature of inhalational anthrax.

Gastrointestinal anthrax is difficult to diagnose because of its rarity and nonspecific symptoms including nausea, vomiting, anorexia, and fever. As the disease progresses, patients often develop acute, severe abdominal pain, hematemesis, and bloody diarrhea. Diagnosis is usually considered only with a history of ingesting contaminated meat in the setting of an outbreak. Microbiological cultures do not help confirm the diagnosis. The diagnosis of oropharyngeal anthrax can be made from the clinical and physical findings in a patient with the appropriate epidemiological history. Sore throat, dysphagia, hoarseness, cervical lymphadenopathy, and edema as well as fever are often presenting symptoms.[194,206,207]

Meningitis resulting from anthrax is clinically indistinguishable from meningitis attributable to other etiologies. An important distinguishing feature is that the cerebral spinal fluid is hemorrhagic in as many as 50% of cases. The diagnosis can be confirmed by identifying the organism in cerebral spinal fluid by microscopy, culture, or both.

Serology is generally only useful in making a retrospective diagnosis. Antibody to PA or the capsule develops in 68% to 93%[208–211] of reported cutaneous anthrax cases and 67% to 94%[210,211] of reported oropharyngeal anthrax cases. A positive skin test to anthraxin (an undefined antigen derived from acid hydrolysis of the bacillus that was developed and evaluated in the former Soviet Union) has also been reported[212] to help with the retrospective anthrax diagnosis. Western countries have limited experience with this test.[213] The Food and Drug Administration (FDA) has recently approved two tests: (1) the QuickELISA Anthrax-PA Kit (Immunetics, Boston, MA) for identification of PA toxin in blood from infected human casualties, and (2) the PCR Joint Biological Agent Identification and Diagnostic System (Idaho Technology Inc, Salt Lake City, UT) anthrax test for rapid identification of bacteria in blood and blood culture samples.[214]

TREATMENT

Cutaneous anthrax without toxicity or systemic symptoms may be treated with oral penicillin if the infection did not originate with a potential aerosol exposure. However, if an inhalational exposure is also suspected, ciprofloxacin or doxycycline is recommended as first-line therapy.[202,203,215] Effective therapy reduces edema and systemic symptoms but does not change the evolution of the skin lesion. Treatment should be continued for 7 to 10 days, unless inhalational exposure is suspected; then treatment should be continued for 60 days. However, recent studies of the 2001 bioterrorism event have identified problems associated with pro-

longed treatment, mass prophylaxis, and medication compliance.[216–221] Amoxicillin is recommended for patients who cannot take fluoroquinolones or tetracycline-class drugs; however, increasing evidence shows that *B anthracis* possesses β-lactamase genes that may reduce the efficacy of this treatment.[222–227] In addition, if a bioterrorism event occurs, the bacterial strains used may be naturally antibiotic resistant or genetically modified to confer resistance to one or more antibiotics.

Tetracycline, erythromycin, and chloramphenicol have also been used successfully[228] for treating rare cases caused by naturally occurring penicillin-resistant organisms. Additional antibiotics shown to be active in vitro include gentamicin, cefazolin, cephalothin, vancomycin, clindamycin, and imipenem.[229–231] These drugs should be effective in vivo, but no reported clinical experience exists. Experimental infections using the inhalational mouse model have demonstrated significant efficacy using these additional antibiotics.

Inhalational, oropharyngeal, and gastrointestinal anthrax should be treated with intravenous therapy using two or more antibiotics. The therapy should initially include a fluoroquinolone or doxycycline with one or more of the following antibiotics: clindamycin, rifampin, penicillin, ampicillin, vancomycin, amino-glycosides, chloramphenicol, imipenem, and/or clarithromycin.[202,215] Tactical Combat Casualty Care guidelines have been established for medical management of patients in chemical, biological, radiological, nuclear, or high-yield explosives environments.[232] The Centers for Disease Control and Prevention issued guidelines for the treatment and management of human anthrax disease.[205,206] New guidelines published in 2014 recommend linezolid over clindamycin—when appropriate—to prevent toxin formation and the use of adjunctive corticosteroids when indicated.[233–236] The World Health Organization has also issued guidelines for the surveillance and control of anthrax in humans and animals and can be accessed at the following website: http://www.who.int/csr/resources/publications/anthrax/WHO_EMC_ZDI_98_6/en/.

Patients often require intensive care unit support, including appropriate vasopressors, oxygen, and other supportive therapy, because of the disease's severity and rapid onset. Recommendations for treatment during pregnancy and for pediatric populations follow similar guidelines.[234–236] The development of severe bacterial sepsis has been well documented for anthrax in both human clinical disease and experimental animal models. The expression of LT and ET as well as other virulence factors such as ALO promote the development of systemic inflammatory response syndrome by both damage-associated molecular patterns and pathogen-associated molecular patterns.[178] This immunologic stimulation, if unregulated or limited, results in the formation of a cytokine cascade and eventual storm resulting in multiorgan system failure and rapid death of humans exposed to inhalational anthrax as well as other select agents. This immunologic over-response has prompted the evaluation of various augmentation therapies to mitigate these events. One such therapy that received FDA approval in 2012 is raxibacumab (GlaxoSmithKline, Brentford, Middlesex, United Kingdom), a human IgG1 monoclonal antibody directed against the PA antigen of *B anthracis*.[237,238] This product was the first monoclonal antibody approved for use in the treatment of severe inhalational anthrax under the FDA's Animal Efficacy Rule.[237–239] The study found that 64% of *Cynomolgus macaque* monkeys and 44% of rabbits with inhalational anthrax survived, whereas all placebo control animals died from both groups.[239] An additional study comparing antibiotics and raxibacumab against antibiotics demonstrated a 82% survival for combination therapy versus 65% for antibiotics only. When rabbits were treated with levofloxacin plus raxibacumab verses levofloxacin alone, the absolute difference in survival rates between the groups was not statistically significant; however, clinically there was only an 18% death rate in the levofloxacin plus raxibacumab group and a 35% death rate in the levofloxacin only group.[240,241]

PROPHYLAXIS

Prophylactic Treatment After Exposure

Experimental evidence[242] has demonstrated that treatment with antibiotics (including ciprofloxacin, doxycycline, and penicillin) beginning 1 day after exposure to a lethal aerosol challenge with anthrax spores can significantly protect against death. Combining antibiotics with active vaccination provides the optimal protection. Recent analysis has suggested postexposure vaccination may shorten the duration of antibiotic prophylaxis, providing the least expensive and most effective strategy to counter a bioterrorism event.[243–245]

Active Immunization

Emergent BioSolutions (Rockville, MD) produces the only licensed human vaccine against anthrax, anthrax vaccine adsorbed (BioThrax). This vaccine is made from sterile filtrates of microaerophilic cultures

of an attenuated, unencapsulated, nonproteolytic strain (V770-NP1-R) of *B anthracis*. The filtrate, containing predominantly 83-kDa PA, is adsorbed to 1.2 mg/mL of aluminum hydroxide in 0.85% sodium chloride. The final product also contains 100 μg/mL of formaldehyde and 25 μg/mL of benzethonium chloride as preservatives. Some vaccine lots contain small amounts of LF and lesser amounts of EF, as determined by antibody responses in vaccinated animals.[246,247] Low levels of antibody to LF and EF by Western blot have been reported in some vaccines, but these did not contribute significantly during toxin neutralization assays.[248] The vaccine is stored at 2°C to 8°C. The vaccine should be given to industrial workers exposed to potentially contaminated animal products imported from countries in which animal anthrax remains uncontrolled. These products include wool, goat hair, hides, and bones. People in direct contact with potentially infected animals and laboratory workers should also be vaccinated. Vaccination is also indicated for protection against anthrax use in biological warfare.

Recommendations have been made for anthrax vaccine use in the United States.[249,250] The current guidelines recommend the anthrax vaccine adsorbed vaccine should be administered to prime the immune system to prevent infection as either a preexposure vaccine or after exposure to aerosolized *B anthracis* pores. For preexposure protection the Advisory Committee on Immunization Practices recommends intramuscular injections starting on day 0 followed by week 4, and every 6 months (6, 12, and 18 months) for a total of 5 doses as the initial vaccination series. Since no in vitro correlate of immunity exists for humans, annual boosters are recommended if the potential for exposure continues. For postexposure to anthrax, those persons who have been previously unvaccinated should receive the vaccine as a three dose, subcutaneous series (at 0, 2, and 4 weeks) in addition to the administration of a 60-day course of an appropriate antimicrobial therapeutic.

More than 2.6 million US military personnel have received the licensed anthrax vaccine adsorbed vaccine, and no unusual rates of serious adverse events have been noted.[251] Additional studies also support the safety of the anthrax vaccine.[252–260] The next generation vaccine, recombinant PA, may afford equivalent protection with a decrease in reactogenicity. A live attenuated, unencapsulated spore vaccine is used for humans in the former Soviet Union. The vaccine is given by scarification or subcutaneously. Its developers claim that it is reasonably well tolerated and shows some degree of protective efficacy against cutaneous anthrax in clinical field trials.[212] New attenuated vaccines developed in the United States are being evaluated for efficacy in inhalational anthrax animal models.[261] Recent studies have demonstrated a fourfold rise in anti-PA immunoglobulin G (IgG) titers of 85% and 100% in adults receiving two and three doses, respectively, of either subcutaneous or intramuscular AVA.[262–265]

One hundred percent of the vaccinees developed a rise in titer in response to the yearly booster dose. When tested by an enzyme-linked immunosorbent assay, the current serologic test of choice, more than 95% of vaccinees seroconvert after the initial three doses.[248]

A rough correlation exists between antibody titer to PA and protection of experimental animals from infection after vaccination with the human vaccine. However, the exact relationship between antibody to PA as measured in these assays and immunity to infection remains obscure because the live attenuated Sterne veterinary vaccine (made from an unencapsulated, toxin-producing strain) protects animals better than the human vaccine, yet it induces lower levels of antibody to PA.[246–248]

A recent study evaluating the response of mice to recombinant PA revealed significant variation of fine specificity of humoral response to the antigen even among genetically identical mice using the same immunogen and environment.[266,267] The authors demonstrated a heterogeneity of response to the PA antigen and identified specific epitopes that correlated to seroconversion and LeTx neutralization. Then they speculated that this observed stochastic variation in humoral immunity was likely a major contributing factor to the heterogeneity of vaccine response. Although these data suggest enhancing immunologic recognition of specific epitopes can improve vaccine protective response, the current anthrax vaccine adsorbed vaccine has demonstrated significant protection to nonhuman primates when exposed to inhalational challenge with large doses of anthrax spores.[268–274]

The protective efficacy of experimental PA-based vaccines produced from sterile culture filtrates of *B anthracis* was clearly demonstrated by various animal models and routes of challenge.[275] A placebo-controlled clinical trial was conducted with a vaccine similar to the currently licensed US vaccine.[276]

This field-tested vaccine was composed of the sterile, cell-free culture supernatant from an attenuated, unencapsulated strain of *B anthracis*, different from that used to produce the licensed vaccine and grown under aerobic, rather than microaerophilic, conditions.[277]

This vaccine was precipitated with alum rather than adsorbed to aluminum hydroxide. The study population worked in four mills in the northeastern United States where *B anthracis*–contaminated imported goat hair was used. The vaccinated group, compared to a placebo-inoculated control group,

was afforded 92.5% protection against cutaneous anthrax, with a lower 95% confidence limit of 65% effectiveness. There were insufficient inhalational anthrax cases to determine whether the vaccine was effective. This same vaccine was previously shown to protect rhesus monkeys and other animal models against an aerosol exposure to anthrax spores.[277–282] No controlled clinical trials in humans of the efficacy of the currently licensed US vaccine have been conducted. This vaccine has been extensively tested in animals and has protected guinea pigs against both an intramuscular[247,248,280] and an aerosol challenge.[246] The licensed vaccine has also been shown to protect rhesus monkeys against an aerosol challenge.[242,270,278,282] The Centers for Disease Control and Prevention issued recommendations on the use of the anthrax vaccine in 2009.[273]

Recombinant PA is undergoing clinical trials and is considered the next-generation anthrax vaccine. Additionally, other nontoxin based vaccine approaches are being explored. These approaches include using the *B anthracis* capsule[270,279,281,283–285] and spore-specific proteins.[286–289] Although these novel antigens have been promising, it is generally agreed that PA will continue to have a prominent role in licensed anthrax vaccines.

Side Effects

In two different studies, the incidence of significant local and systemic reactions to the vaccine used in the placebo-controlled field trial was 2.4% to 2.8%[82] and 0.2% to 1.3%.[277] The vaccine licensed in the United States is reported to have a similar incidence of reactions.[290] Local reactions considered significant include induration, erythema in an area larger than 5 cm in diameter, edema, pruritus, warmth, and tenderness. These reactions peak at 1 to 2 days and usually resolve within 2 to 3 days afterward. Rare reactions include edema extending from the local site to the elbow or forearm, and a small, painless nodule that may persist for weeks. A recent study indicated that administering the vaccine over the deltoid muscle instead of the triceps can significantly reduce the frequency of local reactions.[251]

People who have recovered from a cutaneous infection with anthrax may have severe local reactions from being vaccinated.[276] Systemic reactions are characterized by flu-like symptoms, mild myalgia, arthralgia, headache, and mild-to-moderate malaise that last for 1 to 2 days. No long-term sequelae of local or systemic reactions exist and no suggestion of a high frequency or unusual pattern of serious adverse events exists.[251,256,257,291,292]

SUMMARY

Anthrax is a zoonotic disease that occurs in domesticated and wild animals. Humans become infected by contact with infected animals or contaminated products. Under natural circumstances, infection occurs by the cutaneous route and only rarely by the inhalational or gastrointestinal routes. An aerosol exposure to spores causes inhalational anthrax, which is of military concern because of its potential for use as a biological warfare agent. Aerosol exposure begins with nonspecific symptoms followed in 2 to 3 days by the sudden onset of respiratory distress with dyspnea, cyanosis, and stridor; it is rapidly fatal. Radiography of the chest often reveals characteristic mediastinal widening, indicating hemorrhagic mediastinitis. Hemorrhagic meningitis frequently coexists. Given the rarity of the disease and its rapid progression, it is difficult to diagnose inhalational anthrax. Treatment consists of massive doses of antibiotics and supportive care. Postexposure antibiotic prophylaxis is effective in laboratory animals and should be instituted as soon as possible after exposure. A licensed, antigen-based, nonviable vaccine is available for human use.

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Chapter 7

BRUCELLOSIS

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INTRODUCTION

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SUMMARY

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INTRODUCTION

Brucellosis is a zoonotic infection of domesticated and wild animals caused by bacteria of the genus *Brucella*. Humans become infected by ingesting animal food products directly contacting infected animals or inhaling infectious aerosols either inadvertently or by intentional means through bioterrorism. Brucellosis is currently considered to be one of the world's leading zoonoses.[1]

Military medicine has played a large role in discovering and defining brucellosis in humans.[2] In 1751 G Cleghorn, a British army surgeon stationed on the Mediterranean island of Minorca, described cases of chronic, relapsing febrile illness and cited Hippocrates' description of a similar disease more than 2,000 years earlier.[3] Three additional British army surgeons working on the island of Malta during the 1800s were responsible for important descriptions of the disease. JA Marston described clinical characteristics of his own infection in 1861.[4] In 1887 David Bruce, for whom the genus *Brucella* is named, isolated the causative organism from the spleens of five fatal cases and placed this bacterium within the genus *Micrococcus*.[5] Ten years later, ML Hughes, who had coined the name "undulant fever," published a monograph that detailed clinical and pathological findings in 844 patients.[6]

In that same year, Bernhard Bang, a Danish investigator, identified a bacterium, which he called the "bacillus of abortion," in placentas and fetuses of cattle suffering from contagious abortion.[7] In 1917 Alice C Evans recognized that Bang's organism was identical to that described by Bruce as the causative agent of human brucellosis. The bacterium infects mainly cattle, sheep, goats, and other ruminants, in which it causes abortion, fetal death, and genital infections.[8,9] Humans, who are usually infected incidentally by contact with infected animals or ingestion of dairy foods, may develop numerous symptoms in addition to the usual ones of fever, malaise, and muscle pain. With the worldwide distribution of brucellosis, international travel and military deployments increase the risk of exposure to this disease.[10] In particular, the deployment of US military and coalition forces into Iraq, Afghanistan, Libya, and other Middle Eastern countries has posed particular risk from environmental and food source animals.[11–13] The disease frequently becomes chronic and may relapse, even with treatment. Laboratory-acquired infections have been documented as awareness of this disease has increased,[14–17] and as biodefense research expands in the academic and biotechnology industries, laboratory accidents may unfortunately become more frequent and significant.[18] Strict adherence to proper engineering controls, good laboratory and microbiology techniques, and the use of personal protective equipment significantly reduces the incidence of laboratory-acquired infections.[19,20] No vaccine is available that can safely be used to prevent laboratory-acquired brucellosis.

The ease of transmission by aerosol underscores the concern that *Brucella* might be used as a biological warfare agent. The United States began developing *Brucella suis* as a biological weapon in 1942. The agent was formulated to maintain long-term viability, placed into bombs, and tested in field trials during 1944–1945 with animal targets. By 1969 the United States terminated its offensive program for development and deployment of *Brucella* as a weapon and destroyed all of its biological weapon munitions. Although the munitions developed were never used in combat, studies conducted under the offensive program reinforced the concern that *Brucella* might be used against US troops as a biological warfare agent.[21] Even before the post–September 11, 2001 attacks, civilian populations were recognized as potential high yield targets. In 1997 a model of aerosol attack with *Brucella* on an urban population estimated an economic impact of $477.7 million per 100,000 persons exposed.[22] *Brucella* represents one of many biological agents of zoonotic disease that could pose threats as terrorist weapons against human or agricultural targets.[23] Several reviews that focus on the potential use of the brucellae as agents of bioterrorism or biowarfare have been published.[24–26]

THE INFECTIOUS AGENT

Brucellae are small, nonmotile, nonsporulating, nontoxigenic, nonfermenting, facultatively intracellular, gram-negative bacteria that represent a single "genospecies" from a phylogenetic perspective.[27] However, for epidemiologic purposes and ease and accuracy of communication, *Brucella* strains are classified as separate "nomenspecies" based on readily distinguished phenotypic characteristics that include host specificity.[28] There are presently 10 of these recognized "nomenspecies" (Table 7-1). *Brucella melitensis*, *B suis*, *Brucella abortus*, and *Brucella canis* are the classic causative agents of disease in humans. Human infections with the marine mammal strain *Brucella ceti*[29,30] and a strain (*Brucella inopinata*) of unknown origin[31–33] have also recently been described, but prevalence of such infections is unclear.

Human infections with *Brucella ovis*, *Brucella neotomae*, *Brucella pinnipedialis*, and *Brucella microti* have not been described. Brucellae grow best on trypticase soy-based media or other enriched media with a typical doubling time of 2 hours in liquid culture. Although *B melitensis* bacteremia can be detected within 1 week by using automated culture systems,[34] cultures should be maintained for at least 4 weeks, with weekly subculture, for diagnostic purposes. Most biovars of *B abortus* require incubation in an atmosphere of 5% to 10% carbon dioxide for growth. Brucellae may produce urease and may oxidize nitrite to nitrate; they are oxidase- and catalase-positive. Species and biovars are differentiated by their carbon dioxide requirements; ability to use glutamic acid, ornithine, lysine, and ribose; hydrogen sulfide production; growth in the presence of thionine or basic fuchsin dyes; agglutination by antisera directed against certain lipopolysaccharide (LPS) epitopes; and by susceptibility to lysis by bacteriophage. *Brucella* can grow on blood agar plates and does not require X or V factors for growth.

Serological agglutinating antibodies have been used worldwide as the definitive diagnostic test for brucellosis infection. The standard tube agglutination test is the modified *Brucella* microagglutination test.[35] This test uses direct agglutination of bacterial antigens by specific antibodies of the immunoglobulin (Ig), IgG, and IgA classes. Acute infection is indicated by the presence of antigen-specific IgM antibodies, but these antibodies decline rapidly within weeks of the onset of infection. Chronic or relapsing disease is characterized by elevated or increasing levels of IgG and IgA classes.[36] A four-fold or greater rise in *Brucella* agglutination titers demonstrated between acute and convalescent serum specimens collected at least 2 weeks apart in conjunction with clinically compatible illness is considered a confirmatory test for brucellosis infection. Additional confirmatory tests for infection include the isolation of *Brucella* from clinical specimens or the identification of *Brucella* bacteria in tissue cultures by specific immunohistochemical staining.[37] Although highly sensitive and specific, occasionally false positive tests and cross reactions do occur using *Brucella* antibody tests. The cell wall lipopolysaccharide of the *Brucella* organism is antigenically similar to other gram-negative bacteria. Antibodies to *Moraxella phenylpyruvica*, *Yersinia enterocolitica*, *Escherichia coli* O157, and specific *Salmonella* strains are known to provide false positive reactions.[38,39]

Analysis of fragment lengths of DNA cut by various restriction enzymes has also been used to differentiate brucellae groupings.[33] Single nucleotide polymorphism analyses using real time polymerase chain reaction (PCR) have been used to rapidly identify *Brucella* isolates to the species level.[40] Both the multiple loci variable number of tandem repeat analysis and the Bruce-ladder multiplex PCR assays have been recently used to type a variety of marine *Brucella* isolates and differentiate by biovar typing of *B suis* and *B canis*.[41,42] Recent studies using proteomics, complete genomic sequencing, and multi-locus analysis of variable number tandem repeats have rapidly expanded the information on virulence determinants, identification of pathogenicity islands, and evolutionary relatedness among the *Brucella* species.[43–47] Microarrays have now been developed to phylogenetically classify and forensically identify unknown pathogens as well as genotype *Brucella* species.[48,49] The LPS component of the outer cell membranes of the brucellae is different—both structurally and functionally—from that of other gram-negative organisms.[31,32] For instance, in addition to its capacity to provide resistance to complement and potentially serve as a ligand for binding to host cells, experimental evidence indicates that the O-chain of LPS of "smooth" (fully expressed O-chain versus "rough" strains with substantially reduced or absent O-chain) *Brucella* strains directly interferes with the capacity of host macrophages to process antigens via the major histocompatibility complex class II pathway[50] and influences in the intracellular trafficking of the *Brucella* containing vacuoles in host macrophages preventing their fusion with lysosomes.[51] The chemical compositions of the

TABLE 7-1

TYPICAL HOST SPECIFICITY OF *BRUCELLA* SPECIES

Brucella Species	Animal Host	Human Pathogenicity
B melitensis	Sheep, goats	High
B suis	Swine	High
B abortus	Cattle, bison	Intermediate
B canis	Dogs	Low
B ceti	Dolphins, porpoises	Unknown*
B inopinata	Humans	Unknown*
B pinnipedialis	Seals	Not reported
B ovis	Sheep	Not reported
B neotomae	Rodents	Not reported
B microti	Rodents	Not reported

**B ceti* and *B inopinata* strains have been isolated from human disease, but the importance of these strains as human pathogens is presently unknown.

lipid A and core moieties of the *Brucella* LPS are also distinct from those found in the enteric and many other gram-negative bacteria, and these differences greatly reduce the "recognition" of the brucellae by the Toll-like receptors on host macrophages, which allows these bacteria to induce a dampened inflammatory response and use a "stealthy" approach for establishing infections.[52]

One of the unique features of *Brucella* strains is that unlike most pathogenic bacteria, these bacteria produce relatively few "classical" virulence factors.[53] Probably the most widely studied virulence determinants in the *Brucella* strains are the LPS and the Type IV secretion system.[54] The brucellae use this transport system to secrete effector proteins into the cytoplasm of infected mammalian cells. These effector proteins interfere with the activity of the host cell proteins that control the intracellular membrane trafficking. The net result is that the phagosomes within which the brucellae reside in host macrophages avoid extensive interactions with lysosomes and eventually fuse with the host cell endoplasmic reticulum. The formation of these so-called replicative *Brucella* containing vacuoles (or rBCVs) is essential for the virulence of the naturally occurring smooth *Brucella* strains such as *B melitensis*, *B suis*, and *B abortus*. The capacity of *Brucella* strains to survive and replicate in host macrophages is critical for their virulence. Accordingly, in addition to gene products such as these that overtly interfere with biology of the host cell, the brucellae also produce numerous proteins that allow them to successfully resist the environmental stresses they encounter during their intracellular residence in host macrophages. These stresses include exposure to acidic pH, reactive oxygen species and antimicrobial peptides, and nutrient deprivation.[55]

EPIDEMIOLOGY

Animals may transmit *Brucella* organisms during septic abortion, at the time of slaughter, and in their milk. For infected patients, no conclusive evidence indicates that brucellosis can be transmitted from person to person. The incidence of human disease is thus closely tied to the prevalence of infection in sheep, goats, pigs, and cattle, and to practices that allow exposure of humans to potentially infected animals or their products. In the United States, where all 50 states are considered to be "free" of bovine brucellosis and dairy products are routinely pasteurized, illness occurs primarily in individuals such as veterinarians, shepherds, cattlemen, and slaughterhouse workers who have occupational exposure to infected animals. In many other countries, humans more commonly acquire infection by ingesting unpasteurized dairy products, especially cheese.

Less obvious exposures can also lead to infection. In the United States and Australia, for example, hunters have acquired *B suis* infection from feral swine.[56,57] It was also not uncommon for veterinarians to develop brucellosis after accidental exposure to *B abortus* Strain 19 in the United States when this strain was being used as a live vaccine in cattle.[58] Another bovine vaccine strain, Brucella Abortus Vaccine, Strain RB-51, has been used to eradicate brucellosis from the US livestock herds.[59] Accidental human infections with this vaccine cannot be identified using the standard LPS-based diagnostics assay. Brucellae are also highly infectious in laboratory settings; numerous laboratory workers who culture the organism become infected. Disease with a relatively high proportion of respiratory complaints has also been reported in individuals who have camped in the desert during the spring lambing season.[46] *B canis*, a naturally rough strain that typically causes genital infection in dogs, can also infect humans.[60] Although *B canis* infections were once considered rare, it has become apparent that in some areas of the world these infections were probably unrecognized.[60] In the United States the total number of cases of brucellosis remains very low (0.02 to 0.09 cases per 100,000 person-years).[61,62] A major contributing factor to this low incidence of brucellosis can be attributed to a national eradication campaign to eliminate brucellosis in domestic cattle herds. When implemented the human incidence of disease dropped from a high of 6,321 cases in 1947 to 136 cases in 2001 (0.48 cases per million). These few cases are primarily caused by infections with *B melitensis* and now most human cases are distributed in Hispanic populations residing on either side of the Mexico border.[61] The endemic regions located in Latin America, Europe, Africa, and Asia account for most of the human cases of brucellosis with the highest incidences occurring in the former Yugoslav Republic of Macedonia, Algeria, Peru, Iraq, Iran, Syria, Turkey, Kyrgyzstan, and Mongolia.[61,62] With the improvement of diagnostic methods, ever increasing international tourism, and establishment of new eradication programs, the epidemiology of brucellosis will continue to shift and evolve requiring constant vigilance for new foci of disease. Unfortunately, with the rapidly changing political, international, and financial environments, worldwide eradication of this zoonotic disease will be extremely difficult.

PATHOGENESIS

Brucellae can enter mammalian hosts through skin abrasions or cuts, the conjunctiva, and the respiratory tract, and, unlike enteric pathogens such as *Salmonella* or *Shigella* species that infect the lower gastrointestinal tract, the most likely site of bacterial entry is the mucosae of the upper gastrointestinal tract.[63,64] Organisms are rapidly ingested by polymorphonuclear leukocytes, which generally fail to kill them,[65,66] and are also phagocytosed by macrophages (Figure 7-1). Bacteria transported in macrophages, which traffic to lymphoid tissue draining the upper gastrointestinal mucosa, may eventually disseminate to lymph nodes, liver, spleen, mammary glands, joints, kidneys, and bone marrow. As noted previously, the brucellae are resistant to the microbicidal activity of macrophages, and it is their capacity to survive and replicate for prolonged periods in these phagocytes that underlies their ability to produce chronic infections.[55] Histopathologically, the host cellular response may range from abscess formation to lymphocytic infiltration to granuloma formation with caseous necrosis.[58]

Studies in experimental models have provided important insights into host defenses that eventually control infection with *Brucella* organisms. Serum complement effectively lyses some rough strains (ie, those that lack O-polysaccharide side chains on their LPS), but has little effect on smooth strains (ie, bacteria with a long O-polysaccharide side chain); *B melitensis* may be less susceptible than *B abortus* to complement-mediated killing.[67,68] Administration of antibody to mice before challenge with rough or smooth strains of brucellae reduces the number of organisms that appear in liver and spleen. This effect is caused mainly by antibodies directed against LPS, with little or no contribution of antibodies directed against other cellular components.[69]

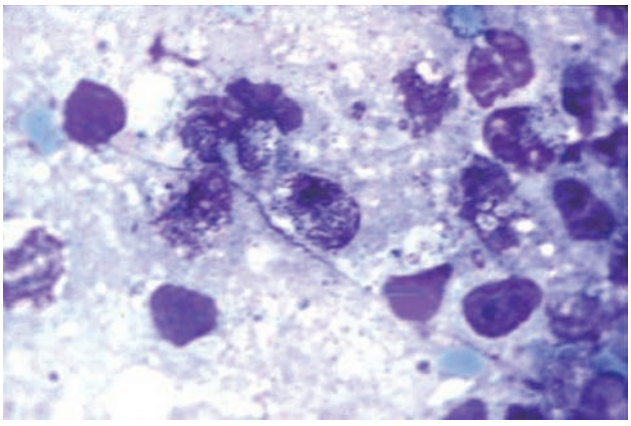


Figure 7-1. Impression tissue smear from a bovine aborted fetus infected with *Brucella abortus*. The bacteria appear as lightly stained, gram-negative cells.
Photograph: Courtesy of John Ezzell, PhD, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

The intensity of an infection in mice can be reduced by transferring from immune to nonimmune animals differentiated $CD4^+$and $CD8^+$ T cells[70] or by the Ig fractions of serum. In particular, the T-cell response to *Brucella* appears to play a key role in the development of immunity and protection against chronic disease.[71,72] Neutralization of *B abortus*-induced host interferon gamma (IFN-γ) during infection in pregnant mice prevents abortion.[73] Moreover, macrophages treated with IFN-γ in vitro inhibit intracellular bacterial replication.[74] Studies in humans support a role for IFN-γ in protection; homozygosity for the IFN-γ +874A allele is associated with about a two-fold increase in incidence of brucellosis.[75] In ruminants, vaccination with live vaccines is required in order to provide protection.[76–78]

These observations suggest that brucellae, like other facultative or obligate intramacrophage pathogens, are primarily controlled by macrophages activated to enhanced microbicidal activity by IFN-γ and other cytokines produced by immune T lymphocytes. It is likely that antibody, complement, and macrophage-activating cytokines produced by natural killer cells play supportive roles in early infection or in controlling growth of extracellular bacteria.

In ruminants, *Brucella* organisms bypass the most effective host defenses by targeting embryonic and trophoblastic tissue. In cells of these tissues, the bacteria grow not only in the phagosome but also in the cytoplasm and the rough endoplasmic reticulum.[79] In the absence of effective intracellular microbicidal mechanisms, these tissues permit exuberant bacterial growth, which leads to fetal death and abortion. In ruminants, the presence of erythritol in the placenta may further enhance growth of brucellae. Products of conception at the time of abortion may contain up to 10^{10} bacteria per gram of tissue.[80] When septic abortion occurs, the intense concentration of bacteria and aerosolization of infected body fluids during parturition often results in infection of other animals and people.

CLINICAL MANIFESTATIONS

Clinical manifestations of brucellosis are diverse and the course of the disease is variable.[81] Patients with brucellosis may present with an acute, systemic febrile illness; an insidious chronic infection; or a localized inflammatory process. However, in the absence of suspicion for brucellosis, many cases seen in the United States are not diagnosed in the early stage of disease, but they are discovered once a focal complication has developed, such as a joint infection. Disease may be abrupt or insidious in onset, with an incubation period of 3 days to several weeks. Patients usually complain of nonspecific symptoms such as fever, sweats, fatigue, anorexia, and muscle or joint aches (Table 7-2). Neuropsychiatric symptoms, notably depression, headache, and irritability, occur frequently. In addition, focal infection of bone, joints, or genitourinary tract may cause local pain. Cough, pleuritic chest pain, and dyspepsia may also be noted. Symptoms of patients infected by aerosol are indistinguishable from those of patients infected by other routes. Chronically infected patients frequently lose weight. Symptoms often last for 3 to 6 months and occasionally for a year or more. Physical examination is usually normal, although hepatomegaly, splenomegaly, or lymphadenopathy may occur. Brucellosis does not usually cause leukocytosis, and some patients may be moderately neutropenic[82]; however, cases of pancytopenia have been noted.[83] In addition, bone marrow hypoplasia, immune thrombocytopenic purpura, and erythema nodosum may occur during brucellosis infections.[84–86] Disease manifestations cannot be strictly related to the infecting species.

Infection with *B melitensis* leads to bone or joint disease in about 30% of patients; sacroiliitis develops in 6% to 15%, particularly in young adults.[87–89] Arthritis of large joints occurs with about the same frequency as sacroiliitis. In contrast to septic arthritis caused by pyogenic organisms, joint inflammation seen in patients with *B melitensis* is mild, and erythema of overlying skin is uncommon. Synovial fluid is exudative, but cell counts are in the low thousands with predominantly mononuclear cells. In both sacroiliitis and peripheral joint infections, destruction of bone is unusual. Organisms can be cultured from fluid in about 20% of cases; culture of the synovium may increase the yield. Spondylitis, another important osteoarticular manifestation of brucellosis, tends to affect middle-aged or elderly patients, causing back (usually lumbar) pain, local tenderness, and occasionally radicular symptoms.[90] Radiographic findings, similar to those of tuberculous infection, typically include disk space narrowing and epiphysitis, particularly of the antero-superior quadrant of the vertebrae, and presence of bridging syndesmophytes as repair occurs. Bone scan of spondylitic areas is often negative or only weakly positive. Paravertebral abscess occurs rarely. In contrast with frequent infection of the axial skeleton, osteomyelitis of long bones is rare.[91]

Infection of the genitourinary tract, an important target in ruminant animals, also may lead to signs and symptoms of disease in humans.[92–94] Pyelonephritis, cystitis, Bartholin's gland abscess and, in males, epididymo-orchitis, may occur. Both diseases may mimic their tuberculous counterparts, with "sterile" pyuria on routine bacteriologic culture. With bladder and kidney infection, *Brucella* organisms can be cultured from the urine. Brucellosis in pregnancy can lead to placental and fetal infection.[95] Whether abortion is more common in brucellosis than in other severe bacterial infections, however, is unknown.

Lung infections have also been described, particularly before the advent of effective antibiotics. Although up to one-quarter of patients may complain of respiratory symptoms, mostly cough, dyspnea, or pleuritic pain, chest radiograph examinations are usually normal.[96] Diffuse or focal infiltrates, pleural effusion, abscess, and granulomas may be noted.

Hepatitis and, rarely, liver abscess also occur. Mild elevations of serum lactate dehydrogenase and alkaline phosphatase are common. Serum transaminases are frequently elevated.[97] Biopsy may show well-formed

TABLE 7-2

SYMPTOMS AND SIGNS OF BRUCELLOSIS

Symptom or Sign	Patients Affected (%)
Fever	90–95
Malaise	80–95
Body aches	40–70
Sweats	40–90
Arthralgia	20–40
Splenomegaly	10–30
Hepatomegaly	10–70

Data sources: (1) Mousa AR, Elhag KM, Khogali M, Marafie AA. The nature of human brucellosis in Kuwait: study of 379 cases. *Rev Infect Dis*. 1988;10:211–217. (2) Buchanan TM, Faber LC, Feldman RA. Brucellosis in the United States, 1960–1972: an abattoir-associated disease, I: clinical features and therapy. *Medicine* (Baltimore). 1974;53:403–413. (3) Gotuzzo E, Alarcon GS, Bocanegra TS, et al. Articular involvement in human brucellosis: a retrospective analysis of 304 cases. *Semin Arthritis Rheum.* 1982;12:245–255.

granulomas or nonspecific hepatitis with collections of mononuclear cells.[81] Spontaneous bacterial peritonitis has also been reported.[98,99]

Other sites of infection include the heart, central nervous system, and skin. *Brucella* endocarditis, a rare, but most feared complication, accounts for 80% of deaths from brucellosis.[100,101] Central nervous system infection usually manifests itself as chronic meningoencephalitis, but subarachnoid hemorrhage and myelitis also occur. Guillain-Barré syndrome has been associated with acute neurobrucellosis and involvement of spinal roots has been noted on magnetic resonance imaging.[102,103] A few cases of skin abscesses have been reported.

DIAGNOSIS

A thorough history that describes details of appropriate exposure (eg, laboratories, animals, animal products, or environmental exposure to locations inhabited by potentially infected animals) is the most important diagnostic tool. The differential diagnosis for brucellosis is broad and includes noninfectious causes such as vasculitis, sacroiliitis, lumbar disk disorders, thrombotic thrombocytopenic purpura, ankylosing spondylitis, abortion complications, depression/suicide, collagen-vascular disease, erythema nodosum, pediatric chronic fatigue syndrome, and malignancy. The infectious disease differential includes fever of unknown origin, rickettsial diseases, bacterial and viral pneumonia, bronchitis, cat scratch fever, cryptococcosis, acute epididymitis, cystitis in females, gastroenteritis, hepatitis, histoplasmosis, infectious mononucleosis, infective endocarditis, influenza, leptospirosis, malaria, meningitis, osteomyelitis, Epstein-Barr virus infection, spontaneous bacterial peritonitis, tuberculosis, tularemia, typhoid fever, and urinary tract infections in men. Brucellosis should also be strongly considered in differential diagnosis of febrile illness if troops have been exposed to a presumed biological attack. PCR and antibody-based, antigen-detection systems may demonstrate the presence of the organism in environmental samples collected from the attack area.

When the disease is considered, diagnosis is based on clinical history, bacterial isolation from clinical samples, biochemical identification of the organism, and by serology. The Centers for Disease Control and Prevention's clinical description of brucellosis is "an illness characterized by acute or insidious onset of fever, night sweats, undue fatigue, anorexia, weight loss, headache and arthalgia."[104] Cultivation of *Brucella* poses a significant hazard to clinical laboratory personnel.[105–108] Rapid detection of the organism in clinical samples using PCR-enzyme-linked immunosorbent assays (ELISAs) or real-time PCR assays can be used to detect *Brucella* DNA in clinical specimens as well as cultivated bacteria and may eventually prove to be the optimal method for identification of these infections.[109–112] Although PCR may have many advantages, a positive PCR is not proof of viable *Brucella*. Many of the assays used are not standardized and have led to false "outbreak" investigations in the United States and, therefore, these assays require proper validation and standardization by the testing laboratory. Typically, the most reliable and simple PCR identification uses a single pair of primers directed against the 16S-23S rRNA operon containing the IS711 or BCSP31 genes.[111] To identify four of the major *Brucella* species, combination primers directed against the BCSP31, OMP3B, OMP2A, and OMP31 external membrane protein genes are used.[111] Multiplex PCR provides a method to identify all known species of *Brucella*. Despite these technical advances, PCR has sensitivity and specificity limitations that depend heavily on the quality of DNA isolated and potential inhibitors present within the clinical samples.[109–111]

According to the Centers for Disease Control and Prevention case definition for brucellosis, the infection may be diagnosed if any of the following laboratory criteria is met:

- isolation of the organism from a clinical specimen;
- fourfold or greater rise in *Brucella* agglutination titer between acute and convalescent-phase serum obtained greater than 2 weeks apart; and
- demonstration by immunofluorescence of *Brucella* in a clinical specimen.[104,112]

Although several serologic techniques such as the Coombs test have been developed and tested, the tube agglutination test remains the standard method.[113] This test, which measures the ability of serum to agglutinate killed organisms, reflects the presence of anti–O-polysaccharide antibody. Use of the tube agglutination test after treating serum with 2-mercaptoethanol or dithiothreitol to dissociate IgM immunoglobulin into monomers makes these antibodies inactive and permits agglutination by immunoglobulin G antibodies that are resistant to dissolution by chemical agents. A titer of 1:160 or higher is considered diagnostic. Most patients already have high titers at the time of clinical presentation, so a fourfold rise in titer may not occur. Immunoglobulin M rises early in disease and may

persist at low levels (eg, 1:20) for months or years after successful treatment. Persistence or increase of 2-mercaptoethanol–resistant (essentially immunoglobulin G) antibody titers has been associated with persistent disease or relapse.[114] Serum testing should always include dilution to at least 1:320, as inhibition of agglutination at lower dilutions may occur. The tube agglutination test does not detect antibodies to *B canis* because this rough organism does not have O-polysaccharide on its surface. Unfortunately, given the need for trained personnel and standardization of the test reagents and control sera, only some references laboratories, such as the Centers for Disease Control and Prevention in Atlanta, Georgia, and the ARUP National Reference Laboratory in Utah, perform the tube agglutination test. ELISAs have been developed for use with *B canis*, but are not well standardized. Although ELISAs developed for other brucellae similarly suffer from lack of standardization, recent improvements have resulted in greater sensitivity and specificity. ELISAs will probably replace the serum agglutination and Coombs tests, thus allowing for screening and confirmation of brucellosis in one test.[115,116]

In addition to serologic testing, diagnosis should be pursued by microbiologic culture of blood or body fluid samples. If unautomated systems are used, blood cultures should be incubated for 21 days, with blind subculturing every 7 days and terminal subculturing of negative blood cultures. For automated systems, incubation of cultures for 10 days with blind culture at 7 days is recommended.[117] Because it is extremely infectious for laboratory workers, the organism should be subcultured only in a biohazard hood. Appropriate personal protective equipment such as a powered air purifying respirator with hood, gown, and gloves should be used when working with cultures or preparing and manipulating bacteria for studies. The reported frequency of isolation from blood varies widely, from less than 10% to 90%; *B melitensis* is said to be more readily cultured than *B abortus*. A recent study indicated that BACTEC™ Myco/F lytic medium (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD), pediatric Peds Plus/F or adult Plus Aerobic/F medium in conjunction with BACTEC™ 9240 blood culture system yielded detection rates of 80% and 100%, respectively.[34] Culture of bone marrow may increase the yield and is considered superior to blood.[118] In addition, direct fluorescent antibody tests under development may offer a method of rapidly identifying these organisms in clinical specimens (Figure 7-2). The case classification of "probable" is defined as a clinically compatible case that is epidemiologically linked to a confirmed case or that has supportive serology (ie, *Brucella* agglutination titer greater than or equal to 160 in one or more serum specimens obtained after the onset of symptoms), and a "confirmed" is a clinically compatible case that is laboratory confirmed.[104,119]

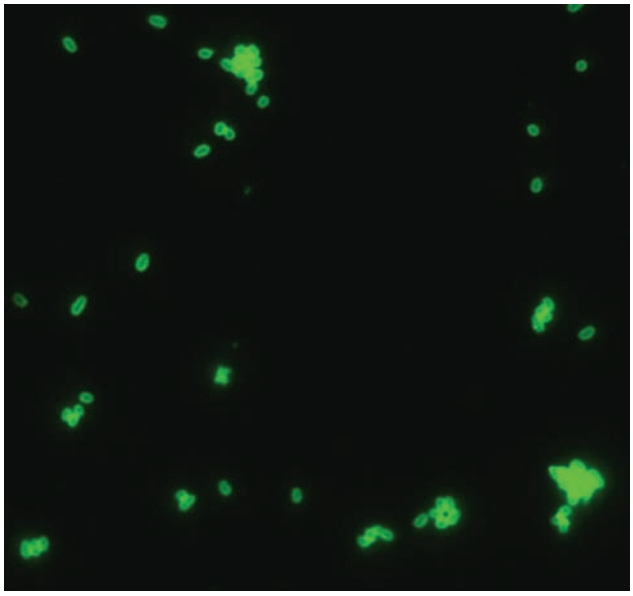


Figure 7-2. Direct fluorescent antibody staining of *Brucella abortus*.
Photograph: Courtesy of Dr John W Ezzell and Terry G Abshire, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

Future trends on rapid identification may use sophisticated protein microarrays to rapidly screen clinical samples or bacterial isolates.[111] However, many of these state-of-the-art identification methods will remain out of reach for resource and fiscally constrained, endemic countries, and thus for many of these areas the primary methods of identification of *Brucella* infections will remain the clinical presentation and traditional diagnostic methods.

TREATMENT

Brucellae are sensitive in vitro to a number of oral antibiotics and to aminoglycosides. In June 2005 at the Clinical Laboratory Standards Institute (CLSI formally known as National Committee for Clinical Laboratory Standards or NCCLS) meeting, the minimum inhibitory concentration breakpoints were established (Table 7-3) for *Brucella* along with the standard procedures for in vitro testing.[120] Therapy with a single drug has resulted in a high relapse rate, so combined regimens should be used whenever possible.[104,121–125] A 6-week regimen of doxycycline (200 mg/day administered orally) and streptomycin (1 g/day administered intramuscularly for

TABLE 7-3

BRUCELLOSIS MINIMUM INHIBITORY CONCENTRATION BREAKPOINT RANGES

Antimicrobial	Minimum Inhibitory Concentration range (μg/mL)
Azithromycin	0.25 – > 64
Chloramphenicol	0.5 – 4
Ciprofloxacin	0.25 – 8
Streptomycin*	≤ 8
Tetracycline	0.03 – 0.5
Doxycycline	≤ 1
Gentamicin	0.5 – 4
Rifampin	< 0.12 – 2
Levofloxacin	< 0.06 – 4
Trimethoprim – Sulfamethoxazole	≤ 2/38

*The streptomycin-susceptible breakpoint is > 16 μg/mL when the test is incubated in CO_2 and > 8 μg/mL when incubated in room air. Data sources: (1) Jorgensen JH. CLSI M45-A2: Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria; Approved Guideline-Second Edition, M45A2. 2010, Clinical and Laboratory Standards Institute, ISBN(s):1562387324. (2) Patel J, Heine H, oral personal communication between these principal investigators at the Clinical and Laboratory Standards Institute Guideline Meeting, June 2005.

the first 2 to 3 weeks) is effective therapy for adults with most forms of brucellosis.[125,126] However, a randomized, double-blind study using doxycycline plus rifampin or doxycycline plus streptomycin demonstrated that 100 mg twice daily oral doxycycline plus 15 mg/kg body weight of oral rifampin once a day for 45 days was as effective as the classical doxycycline plus streptomycin combination, provided these patients did not have evidence of spondylitis.[127] A 6-week oral regimen of both rifampin (900 mg/day) and doxycycline (200 mg/day) is an effective therapeutic treatment with a relapse rate lower than 10%.[128] Several studies, however, suggest that treatment with a combination of streptomycin and doxycycline is more successful and may result in less frequent relapse than treatment with the combination of rifampin and doxycycline.[126–130] Although it is a highly effective component of therapy for complicated infections, streptomycin has disadvantages of limited availability and requirement for intramuscular injection. Other aminoglycosides (netilmicin and gentamicin), which can be given intravenously and may be more readily available, have been substituted for streptomycin with success in a limited number of studies.[97] Fluoroquinolones in combination with rifampin have demonstrated efficacy similar to the doxycycline-rifampin regimen and may replace doxycycline plus rifampin due to potential doxycycline–rifampin interactions.[125,131–134]

Endocarditis may best be treated with rifampin, streptomycin, and doxycycline for 6 weeks; infected valves may need to be replaced early in therapy.[125,135] However, if patients do not demonstrate congestive heart failure, valvular destruction, abscess formation, or a prosthetic valve, conservative therapy with three antibiotics—(1) doxycycline, fluoroquinolone and trimethoprim/sulfamethoxazole, (2) tetracycline or doxycycline plus rifampin, and (3) aminoglycoside or trimethoprim/sulfamethoxazole—may be effective therapy.[136] Patients with spondylitis may require treatment for 3 months or longer. Central nervous system disease responds to a combination of rifampin and trimethoprim/sulfamethoxazole, but patients may need prolonged therapy. The latter antibiotic combination is also effective for children younger than 8 years old.[137] The Joint Food and Agriculture Organization–World Health Organization Expert Committee recommends treating pregnant women with rifampin.[128]

Organisms used in a biological attack may be resistant to these first-line antimicrobial agents. Medical officers should make every effort to obtain tissue and environmental samples for bacteriological culture, so that the antibiotic susceptibility profile of the infecting brucellae may be determined and the therapy adjusted accordingly.

PROPHYLAXIS

To prevent brucellosis, animal handlers should wear appropriate protective clothing when working with infected animals. Meat should be well cooked; milk should be pasteurized. Laboratory workers should culture the organism only with appropriate biosafety level 2 or 3 containment, depending on the stage of bacterial identification (diagnostic sample verses isolated culture).[138] Chemoprophylaxis is not generally recommended for possible exposure to endemic disease.

In the event of a biological attack, the M40 mask (3M, St Paul, MN) should adequately protect personnel from airborne brucellae, as the organisms are probably unable to penetrate intact skin. After personnel have been evacuated from the attack area, clothing, skin, and other surfaces can be decontaminated with standard disinfectants to minimize risk of infection by accidental ingestion, or by conjunctival inoculation of viable organisms. A 3- to 6-week course of therapy with one of the treatments listed above should be considered after a confirmed biological attack or an accidental exposure in a research laboratory.[138,139] There is no safe and effective vaccine currently available to use in humans.

SUMMARY

Brucellosis is a naturally occurring disease in a wide variety of wild and domestic mammals. Although humans are not natural hosts for *Brucella* strains, they can be infected by ingesting contaminated foods (oral route) or slaughtering infected animals (percutaneous route). The brucellae are highly infectious by the airborne route, and this is the route of infection that is presumed to be of the biggest threat to military personnel. Laboratory workers can easily become infected when *Brucella* cultures are handled outside of a biosafety cabinet. Individuals presumably infected by aerosol have symptoms indistinguishable from patients infected by other routes: fever, chills, and myalgia are most common.

Because the brucellae disseminate throughout the reticuloendothelial system, they may cause disease in virtually any organ system. Large joints and the axial skeleton are favored targets; arthritis appears in approximately one-third of patients. Fatalities occur rarely, usually in association with central nervous system or endocardial infection.

Serologic diagnosis uses an agglutination test that detects antibodies to LPS. This test, however, is not useful to diagnose infection caused by *B canis*, a naturally O-polysaccharide-deficient strain. Although ELISAs can more easily be standardized and performed in most clinical laboratories, these tests tend to have a higher degree of false-positive results,[139] and therefore the Rose Bengal (slide-type) agglutination test[140] or Brucella microagglutination test[141] continue to be considered the gold standards for diagnosis. Infection can be most reliably confirmed by culture of blood, bone marrow, or other infected body fluids, but the sensitivity of culture varies widely.

Nearly all patients respond to a 6-week course of oral therapy with a combination of rifampin and doxycycline; fewer than 10% of patients relapse. Alternatively, doxycycline plus fluoroquinolone may be as effective for treating this disease. Six weeks of doxycycline plus streptomycin for the first 3 weeks is also effective therapy; the limited availability of streptomycin may be overcome by substitution of netilmicin or gentamicin. No vaccine is available for humans.

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Chapter 8

GLANDERS

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INTRODUCTION

MILITARY RELEVANCE

HISTORY

INFECTIOUS AGENT

DISEASE
- **Epidemiology**
- **Transmission**
- **Pathogenesis**
- **Clinical Disease in Animals**
- **Clinical Disease in Humans**
- **Laboratory Diagnosis**
- **Treatment**
- **Prophylaxis**

SUMMARY

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INTRODUCTION

Glanders is a debilitating and often fatal zoonotic disease of solipeds including horses, mules, and donkeys caused by infection with the bacterium *Burkholderia mallei*. It is characterized by ulcerating granulomatous lesions of the skin and mucus membranes. Disease progression and pathology in humans and horses are similar, yet the clinical presentation of any two cases in the same species—even if related by direct transmission—may vary significantly.[1–5] Generalized symptoms include fever, myalgia, headache, fatigue, diarrhea, and weight loss. After infection, the organism travels through lymph channels first to regional lymph nodes often causing irritation (lymphangitis, lymphadenitis) en route. Unchecked, organisms may enter the bloodstream and be carried throughout the body. Without effective treatment, the course of disease may range from one that is acute and rapidly fatal to one that is very slow and protracted with alternating remissions and exacerbations.

Glanders is an old disease, having been described toward the beginning of recorded history. It is less commonly known by other names, including equinia, malleus, droes, and farcy. Farcy is an ancient term given to a particular cutaneous manifestation of glanders that at the time (before 1882) was believed to be a completely separate disease in horses. With this cutaneous manifestation of glanders, nodular abscesses (farcy buds) became ulcerated, and regional cutaneous lymphatic vessels became thickened and indurated (farcy pipes) and oozed a glanders-typical yellow-green gelatinous pus (farcy oil).[6] Pure farcy without ulceration of the mucous membranes is rare, if not just a temporary stage, as is vice versa.[3] Humans, goats, dogs, cats, rabbits, and carnivorous predators living in close proximity to infected equids or carcasses have been naturally infected.[1,7] Camels have also been infected and are associated with human disease.[7] Naturally occurring glanders has been eradicated in most countries, but is still found in parts of Africa, the Middle East, Eastern Europe, Asia, and South America. Glanders has drawn interest as a possible warfare agent in the biological weapons programs of several countries.

MILITARY RELEVANCE

B mallei was one of the first biological warfare agents used in the 20th century. Germany used an ambitious biological sabotage campaign in several countries, including the United States, Russia, Romania, France, and Mesopotamia, on both the western and eastern fronts during World War I. Additionally, cattle, horses, mules, and other livestock being shipped from the United States to the Allies were beleaguered and inoculated with cultures of *B mallei*.[8] In 1914, a member of the German army named Anton Dilger, an American-educated surgeon, was sent home to live with his parents in Virginia after a nervous breakdown. He brought strains of *Bacillus anthracis* and *B mallei* and set up a laboratory with his brother's help to grow the organisms in a private home in Chevy Chase, Maryland. Organisms were delivered to another contact from Germany waiting in Baltimore, who then inoculated horses awaiting shipment to the Allies in Europe.

German agents also infected 4,500 mules in Mesopotamia with glanders, a German agent was arrested in Russia with similar intentions in 1916, and French cavalry horses were also targets for intentional glanders infection.[9] Germany and its allies infected many mules and horses on Russia's eastern front, and this action successfully impaired artillery movement and troop and supply convoys. Concurrent with this rise in animal cases during and after the war, human cases increased in Russia. Attempts to contaminate animal feeds also occurred in the United States. Between 1932 and 1945 the Japanese used *B mallei* to deliberately infect horses, civilians, and prisoners of war at the Ping Fan Institute, also known as Unit 731, in occupied Manchuria. Two laboratory workers accidentally exposed to *B mallei* died at the institute in 1937.[10]

In response to perceived biological warfare threats from Japan and Germany, the United States began work on biological warfare agents at Camp Detrick, Maryland (now Fort Detrick) in 1942. *B mallei* was studied for potential use but was not weaponized. Between November 1944 and September 1953, seven laboratory-acquired human infections from *Malleomyces mallei* (the taxonomic name of glanders at that time) occurred in Camp Detrick employees. Howe and Miller reported the first six of these infections in a case series, which remains the largest reported human case series in US medical literature.[5] Information on the seventh case was not published before 2005. All seven original case files were thoroughly reviewed for this chapter. An eighth laboratory-acquired infection occurred in March 2000 during US defensive research on *B mallei*.[11] Also, the Soviets were alleged to have used weaponized *B mallei* against opposition forces in Afghanistan between 1982 and 1984.[12]

The United States signed the Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological and Toxin Weapons, which banned development, production, stockpiling, acquisition, and retention of biological agents, toxins, and the weapons to deliver them in 1972.[9] All offensive biological warfare work at Fort Detrick had ceased by this time; any remaining biological weapons were destroyed by 1973. Biodefense related research aimed toward the development of countermeasures to combat *B mallei* infections, however, continues to be conducted in the United States. A report by the Monterey Institute of International Studies states that between 1931 and 1945 Japan developed *B mallei* as a biowarfare agent. There are no known current attempts for acquisition and use by terrorists.[13]

B mallei was considered a potential biothreat agent in 1947 because of its high infectivity, high degree of incapacitation among those infected, and agent availability.[14] It could be a more significant threat if weaponized. As exemplified by past clusters of laboratory-acquired infections, *B mallei* is particularly infectious by the respiratory route. It is not considered to be highly contagious among humans, and reports of person-to-person transmission are rare. If a determined bioterrorist gained access to the agent, whether from an infected animal, laboratory culture, or commercial culture, the consequences could be severe. Because the clinical symptoms of glanders are protean and nonspecific, and most physicians in the west are not familiar with the disease, diagnosis and treatment may be delayed postattack, even in regions with the most advanced medical facilities. Delayed diagnosis and treatment would likely result in significant morbidity and mortality. In addition, treatment may be complicated by the relative scarcity of knowledge and experience in therapy. As equids and some other animals are susceptible, further spread from animals to humans may continue long after an initial attack. Fortunately, glanders is curable and postexposure prophylaxis may be an option if an attack was rapidly confirmed. As with other agents, genetic engineering could be used to produce a strain with unpredictable virulence and atypical antibiotic resistance. Thus, if *B mallei* was cultured, concentrated, and delivered as an infectious aerosol, significant casualties could result.[15]

HISTORY

Glanders is one of the oldest documented infectious diseases with symptoms being recorded by Hippocrates as early as 425 BCE. Aristotle described the disease in horses in 330 BCE and named it "malleus," meaning hammer or mallet. It was associated with clustered horses around the globe, particularly army horses and mules. The occurrence of glanders in domesticated equids was so familiar that horses and their glanders commonly appeared together in early literature. By about the 4th century, Apsyrtus and Vegetius recognized the contagious nature of the disease and recommended isolation of affected animals. Glanders was not studied in a systematic matter until centuries later. The first veterinary school was established in Lyon, France, in the mid-1700s to deal with the serious problems of rinderpest and glanders.[16] Many researchers at the school became infected and died of glanders during their studies. The first account of humans glanders was not published until 1821.[3] In 1837, Rayer proved the transmissibility of the disease by successfully infecting a horse using material taken from a pustule of a human glanders patient.[17,18] In 1882, Loeffler and Schutz isolated the causative agent, now called *Burkholderia mallei*, in pure culture from the liver and spleen of a glanderous horse.[1,2]

Up until the industrial revolution, horses and mules were the primary modes of transportation in all developing economies. Particularly in urban locations, these animals were housed under crowded conditions, and glanders was passed from the infected to the uninfected. Horses and mules were in high demand during the American Civil War. Thousands of animals passed through remount stations where glanders was found in epidemic proportions. The problem was exacerbated after the American Civil War when infected military stock was sold to civilians, which facilitated spread of the disease to communities. Heavy losses of horses and the infrequent but deadly transmission to humans in the late 19th century led several countries to consider glanders control and eradication programs. Early programs in some countries involved destroying only clinically ill equids, with compensation, and meticulous disinfection of the premises of such cases. Despite these tactics, glanders would reemerge in new or remaining animals in stables and barns that once housed infected animals and the number of countrywide cases increased. The notion of a carrier-state began to be accepted. In spite of epidemic disease in equine populations, there were no simultaneous epidemics in humans.

Vaccines and therapeutic agents were developed but they did not reduce the glanders burden. By 1890, the mallein diagnostic skin test was developed. Control and eradication programs would soon incorporate the testing of all contact equids, followed by quarantine and a recommendation for slaughter of all skin test-positive animals. This program failed in some locales at first because of lack of enforcement and lack

of incentive to owners for killing their nonclinically ill animals. Some horse owners would deliberately hide contact animals to avoid testing, or they would sell these and asymptomatic test-positive animals to unsuspecting individuals to salvage economic loss.[4] Inexpensive steam transportation helped the disease spread by shipping *B mallei*-infected animals to other regions and countries. The United States was blamed for the import of glanderous horses to Cuba in 1872 and for the great increase of glanders cases in Canada near the turn of the 20th century, where tens of thousands of US horses were shipped annually.[3,4]

Once control programs offered indemnity to test-positive and contact animals, and popular belief accepted the existence of a carrier-state, glanders eradication progressed more rapidly. Eliminating glanders in livestock effectively also eradicated the disease in humans in countries with such programs. Great Britain's experience with the rise and fall of glanders outbreaks in equids typifies many countries, and is shown in Figure 8-1.[19] Eradication of glanders was achieved in Great Britain by 1928 and in Canada by 1938, about 30 years after eradication programs were initiated.[20] Glanders was successfully eradicated in the United States by 1942; the last naturally occurring human case was recorded in 1934.[21,22]

Glanders is a zoonotic disease of concern internationally and is notifiable to the 164-member Office International des Epizooties in accordance with the International Animal Health Code.[23] Eradication programs still exist for several countries attempting to eliminate the disease. In more than 500,000 equids tested in Turkey between 2000 and 2001, for example, less than 2% tested positive and were destroyed. Only one of these, a mule, showed clinical signs of infection. Over the past two decades, glanders in livestock was reported in Afghanistan, Bahrain, Belarus, Bolivia, Brazil, China, Eritrea, Ethiopia, India, Iran, Iraq, Kuwait, Latvia, Lebanon, Mongolia, Myanmar, Pakistan, Russia, Turkey, and the United Arab Emirates.[21,24–35] Between 1996 and 2003 glanders in humans was reported in Cameroon, Curacao, Sri Lanka, Turkey, and the United States (laboratory acquired).[21] Exhibit 8-1 depicts the year equine glanders was last reported to

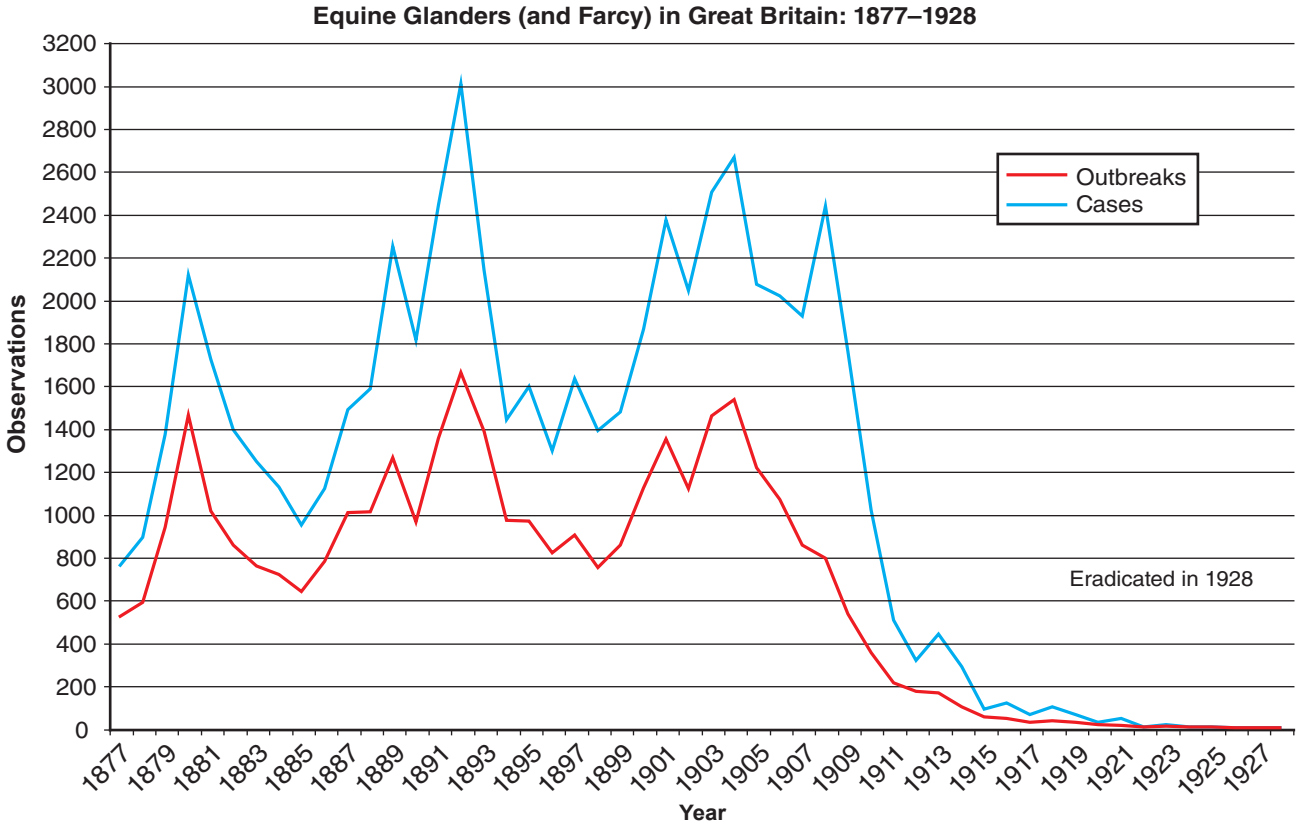


Figure 8-1. Glanders cases and outbreaks reported to the Department for Environment, Food, and Rural Affairs in Great Britain, 1877–1928. Glanders was eradicated in Great Britain in 1928.
Data source: http://www.defra.gov.uk/animalh/diseases/notifiable/glanders/index.htm.

EXHIBIT 8-1

YEAR EQUINE GLANDERS LAST REPORTED TO OIE BEFORE 1996*

Country or Territory	Year	Country or Territory	Year
Australia	1891	Moldavia	1957
Austria	1952	Nambia	1925
Bulgaria	1954	Netherlands	1957
Canada	1938	Northern Ireland	1910
Croatia	1959	Norway	1889
Denmark	1928	Poland	1957
Egypt	1928	Portugal	1952
Estonia	1945	Romania	1960
Finland	1943	Serbia and Montenegro	1959
France	1965	Slovakia	1954
Georgia	1960	South Africa	1945
Germany	1955	Spain	1956
Great Britain	1928	Sudan	1989
Greece	1965	Sweden	1943
Hungary	1956	Switzerland	1937
Ireland	1920	United States of America	1942
Israel	1951	Yug Rep of Macedonia (former)	1957
Japan	1935	Zimbabwe	1911

*The most recent year evidence of equine glanders was reported to the OIE among countries and territories free of equine glanders for at least 5 years (between 1996 and 2013). Included only are territories for which data exist on the reporting of equine glanders to the OIE.
OIE: Office International des Epizooties

the Office International des Epizooties among countries and territories that have been without glanders activity (by Office International des Epizooties report) since 1996. Given the recent outbreaks in horses, donkeys, and dromedaries in some regions of India, Bahrain, Brazil, Lebanon, Pakistan, and the United Arab Emirates, glanders is currently considered a re-emerging infectious disease in these areas.[24–26,28,30–32,34–37] Bioterrorism should be considered as a possible source in the event that confirmed human glanders occurs, in the absence of infected animals, in the countries and territories listed in Exhibit 8-1.

INFECTIOUS AGENT

Glanders is caused by *B mallei*, a gram-negative bacillus that is a close relative to *Burkholderia pseudomallei*, the etiologic agent of melioidosis. Whole-genome comparisons of *B pseudomallei* and *B mallei* in combination with multilocus sequence typing (MLST) analyses suggest that *B mallei* is a clonal descendant of *B pseudomallei* that has evolved through genome downsizing.[38] Unlike *B pseudomallei*, which can be isolated from tropical soil, *B mallei* is an obligate animal pathogen and has not been found free-living in the environment.[39] The lack of flagellar-based motility is a primary means by which *B mallei* can be differentiated from *B pseudomallei*. Growth requirements are not complex and *B mallei* can be cultivated on basic nutrient medium. However, glycerol or glucose can be added to the medium to enhance growth. When stained, the cells typically exhibit bipolar staining.

B mallei is well traveled taxonomically. Since its discovery, this microorganism has been placed in several genera, including *Bacillus*, *Corynebacterium*, *Mycobacterium*, *Loefflerella*, *Pfeifferella*, *Malleomyces*, *Actinobacillus*, and *Pseudomonas*, and was finally assigned to the genus *Burkholderia* in 1992.[40,41] This microorganism is not particularly hardy in the environment.[40] *B mallei* is susceptible to drying, heat, and sunlight. In warm and moist environments, the organism may survive a few months and can survive in room temperature water as long as 1 month.[1,19,42] Experimentally and under the most favorable temperature and moisture conditions, *Loeffler* was able to extend the viability of *B mallei* to 100 days. Survival of *B mallei* in distilled water in the laboratory was determined to be less than 30 days.[43] In nature, viability of the organism is unlikely after 90 days, and most infectivity is lost within 3 weeks.

Particularly in culture, *B mallei* is easily aerosolized as demonstrated by at least seven of the eight laboratory-acquired infections in the United States since 1944. Given its high infectivity by aerosol, laboratory studies on this Centers for Disease Control and Prevention Tier 1 select agent are performed at biosafety level 3 (BSL-3) facilities. Varying degrees of virulence among strains have been shown in the laboratory and in nature.[4,5,7] The infectious dose is considered to be low, depending on the route of infection, susceptibility, and strain virulence. One to 10 organisms of some strains by aerosol are lethal to hamsters.[5] Inhaling only a very few organisms may cause disease in humans, equids, and other susceptible species.[44–46]

DISEASE

Epidemiology

Naturally acquired cases of glanders in humans or equines are sporadic and rare; most countries have eradicated glanders. Glanders is still infrequently reported in northern Africa, the Middle East, South America, Asia, and eastern Europe.[21] Serologic cross-reactivity with *B pseudomallei* precludes the accurate distribution and prevalence of *B mallei* by serologic means alone. However, new reagents and assays potentially leading to improved serodiagnosis of human glanders have been described, as detailed below. Although human outbreaks have been reported in Austria and Turkey, no human epidemic has been recorded.[47]

In nature, chronically infected horses are considered to be the reservoir of *B mallei*, and they may also serve as amplifying hosts. A disease of primarily solipeds, donkeys are considered most prone to develop acute forms of glanders, whereas horses are more prone to develop chronic and latent disease. Mules, a crossbred animal resulting from the mating of horse and donkey, are susceptible to both acute and chronic disease as well as latent infections.[40,48,49] A recent report indicates that Old World camels (dromedaries) can acquire glanders naturally when kept in close proximity to infected horses.[35] Clinical disease in dromedaries closely resembled that seen in equids. Humans are an accidental host.

Zoonotic transmission of *B mallei* from equid to human is uncommon, even with close and frequent contact with infected animals, which may be explained by low concentrations of organisms from infection sites and a species-specific difference in susceptibility to virulent strains. During World War II human glanders was rare despite a 30% prevalence in horses in China.[50] Between 5% and 25% of tested animals in Mongolia were reactive, yet no human cases were reported. With successful transmission, however, humans are susceptible to infection.

Humans exposed to infected equids have contracted glanders in occupational, hobby, and lifestyle settings. Naturally infected humans have included veterinarians and veterinary students, farriers, flayers (hide workers), transport workers, soldiers, slaughterhouse personnel, farmers, horse-fanciers and caretakers, and stable hands. Subclinical or inapparent infections in horses and mules have posed a hidden risk to humans. Infection by ingesting contaminated food and water has occurred; however, it does not appear to be a significant route of entry for infections in humans.[1,7,51] Laboratory workers have also been rarely and sporadically infected. In contrast to zoonotic transmission, culture aerosols are highly infectious to laboratory workers. The six infected workers in the Howe and Miller case series represented 46% of the personnel actually working in the laboratories during the year of occurrence.[5]

Different strains of *B mallei* can now be discriminated by multiple-locus variable number tandem repeat analysis (MLVA), MLST, random amplified polymorphic DNA, and other fingerprinting methods. Such procedures have been shown to be useful in tracking the source and spread of an outbreak strain and in geographic/clonal relationship studies, and they have been described and summarized elsewhere.[24,52] For example, Godoy et al used MLST with a set of seven loci in epidemiologic studies analyzing many isolates of *B mallei*, *B pseudomallei*, and *Burkholderia thailandensis* (a closely related but nonpathogenic environmental species) from diverse geographical locations that represented 71 sequence types; specific clones isolated from animals that were associated with disease in humans were identified.[35,53] MLST was most useful for distinguishing strains of *B pseudomallei* and *B thailandensis*, which were clearly distinguished by the divergence between the alleles of seven loci. However, all the geographically diverse isolates of *B mallei* analyzed had identical allelic profiles that clustered within the *B pseudomallei* group of isolates; alleles at six of the loci in *B mallei* were also present in *B pseudomallei* isolates, and the allele at the seventh locus in *B mallei* differed at only a single nucleotide site from *B pseudomallei*. *B mallei* was considered to cluster within and to be a clone of *B pseudomallei* instead of a separate species. However, one recent analysis of camel-associated glanders used one *B mallei*-specific sequence type to confirm the laboratory identification of glanders.[35]

MLVA has been found to be more useful for subtyping different strains of *B mallei*. For example, an MLVA analysis of a *B mallei* strain isolated from a diseased camel in Bahrain revealed close genetic proximity to a specific strain, which caused an earlier outbreak of glanders in horses in the United Arab Emirates in 2004.[35] The MLVA was based on 23 different loci, as reported previously.[28] Similar analyses focused on *B mallei* isolates from the Punjab region of Pakistan demonstrated that these strains were genetically distinct from isolates from other countries.[28] In the event of a deliberate release of *B mallei* or a focal outbreak of glanders in humans or animals, these types of analyses would be critical tools for facilitating investigations aimed at determining the source of the organism.

Transmission

Transmission is direct by bacterial invasion of the nasal, oral, and conjunctival mucous membranes; by inhalation into the lungs; and by invasion of abraded or lacerated skin. Areas of the body most often exposed include the arms, hands, and face. Considering the affinity for warm and moist conditions, *B mallei* may survive longest in stable bedding, manure, feed and water troughs (particularly if heated), wastewater, and in enclosed equine transporters.[1] Transmission has occurred via handling contaminated fomites such as grooming tools, hoof trimming equipment, harnesses, tack, feeding and husbandry equipment, bedding, and veterinary equipment. Such equipment stored away from any contact with equids for at least 3 months, even without disinfection, is not likely to be an infection source.

Reports of the circumstances surrounding zoonotic transmission are diverse. Here are a few examples:

- equids snorting in the vicinity of humans or human food;
- the wiping of equine nasal exudate off a human arm with a blade of grass (local infection occurred at wipe site);
- sleeping in the same barn or stall as apparently healthy equids;
- accidental puncture with contaminated equipment;
- wiping an eye or nostril after contact with an equid;
- being licked by a glandered horse; and
- stall cleaning without any direct equine contact.[3,54,55]

The nature of much of the work in horse handling is physical, often producing skin abrasions under normal circumstances. Although absorption through intact skin is believed to be unlikely, patients may insist their skin was intact at the time of exposure. Among 105 chronic human cases associated with equid exposure described by Robins, only 40 (38%) reported a wound present.[3] In 27 cases (17%) the absence of a wound was specifically noted.[3]

Laboratory infections have followed procedures that involved washing and aeration of cultures. Air samples and swabs from equipment, tables, and benches failed to detect residual contamination in laboratories after the six US laboratory-acquired events that occurred between 1944 and 1945. Seven of the eight Fort Detrick laboratory-acquired infections also occurred at a time when mouth-pipetting was common practice. The first six patients acknowledged using this technique to clear blocked pipettes and to blow contents out of pipettes that were calibrated to the tip. The eighth case patient involved a microbiologist who had 2 years of experience of working with *B mallei* in BSL-3 containment but did not always wear latex gloves.[11,56] Based on the clinical manifestation of unilateral axillary lymphadenopathy, transmission in this case was believed to be percutaneous, yet a break in the skin or a specific exposure-associated laboratory incident was not recalled. This is not surprising as most laboratory-acquired infections are not associated with injury, or a recollection of injury.[57] This patient had a 13-year history of diabetes, however, and collected blood via finger-stick morning and evening. It is possible that a recent finger-stick site may have been a potential entry point. Bacterial surveys of the laboratory found no contamination, and all engineering controls were validated as functional.

Human-to-human transmission is rare, but it has been reported. The majority of documented events were in medical practice, at autopsy, in the diagnostic laboratory, and in patient care settings before clearer understanding of universal precautions existed.[1,3,11] Transmission also occurred in home settings. Close contact while caring for glanders-infected individuals at home led to infecting other family members.[3] At least one entire family became infected. In this case, two children and the wife of a chronically infected stable hand contracted glanders. The wife was presumably infected sexually; the 4-year-old child was likely infected by close contact with a 2-year-old sibling who was presumably infected by one of the parents. Robins found that among the 156 chronic infections he studied, 10% were directly caused by another human case.[3]

Human infection by ingestion has not been definitively reported. Stomach contents were found to inactivate *B mallei* experimentally in 30 minutes.[47] In his detailed 1886 report on the etiology of glanders, Loeffler describes several accounts of feeding meat

from glanderous horses to humans without causing disease.[1] In one account, more than 100 glanderous horses were slaughtered and fed to soldiers without incident. Although not clear in his report, it is most likely that in these cases the meat was cooked just as was customary for a military mess at that time. In another case, consumption of raw glanderous meat by a veterinarian seeking to answer the ingestion question did not produce disease. An 1866 veterinary journal report, however, describes two persons who contracted glanders after consuming milk from a glanderous mare. Because these individuals were also exposed to the mare, infection by ingestion could not be determined.[1]

Monogastric animals, including the lion, tiger, domestic cat, dog, and bear, became infected with *B mallei* after ingesting raw meat.[1] Regarding wild animals, Loeffler posited that crunching bones might cause enough oral trauma to introduce the organism through defects in the oral mucosa rather than by entry through the healthy digestive tract.[1] This explanation, however, does not explain infections in dogs, domestic cats, and captive wildlife that were fed only boneless meat from glanderous horses. In 2010, four lions and one tiger at a zoo in Tehran died from glanders.[29] Although not definitively proven, this outbreak was attributed to ingestion of soliped meat that had not been screened for *B mallei*. Based on this limited collection of testimonies and the current understanding of glanders pathogenesis, one may infer that ingestion of the live organism by humans is unlikely to cause disease.

These features of transmission exemplify the requirement for BSL-3 containment and safety practices when working with *B mallei*. Adherence to safety procedures and universal barrier precautions is also prudent. In the presence of potentially infected equids, transmission risk is also reduced by universal precautions as well as procedures that reduce inhalation risk of potentially contaminated aerosols. Advances in medicine, infection control, and therapeutics make it less likely today than 100 years ago for human-to-human transmission to occur even in the event of a human outbreak, whether related to bioterrorism or not. It is also highly unlikely that an equid reservoir would become established. Acute disease is expected to manifest in a significant proportion of exposed equids, which would allow emergency response, quarantine, traceback, and eradication procedures. Long-term exposure to asymptomatic chronically infected equids that evade detection and are handled without precautions could become a sporadic but perilous risk to humans, and less caution may be used around them.[3]

Among equids, transmission is primarily by oronasal mucous membrane exposure, inhalation, and mastication (possibly ingestion) of skin exudates and respiratory secretions of infected animals, including those with latent and subclinical infection. The sharing of feed and water troughs facilitates this, as do common equid behaviors that include grooming and snorting.[40,48,49] Since equids cannot breathe through their mouths, simple exhalation and snorting to clear nasal passages serve to finely aerosolize infectious nasal efflux from an infected equid, which poses a transmission risk to susceptible hosts (including humans) in the vicinity.

Transmission through ocular mucous membranes and abrasions in the skin is also possible. Vertical transmission from mare to foal has occurred naturally in horses. In utero transmission from sow guinea pig to pup has also occurred in housed laboratory animals.[1] Sexual transmission from stallion or jack to mare or jenny has also been observed. The breeding of asymptomatic stallions was responsible for some glanders spread near the turn of the 20th century.[4]

Carnivores can become infected after eating contaminated carcasses and meat.[29,58] Reported outbreaks in captive wild felids suggest that they appear to be more susceptible than canids.[40,48,58,59] Glanders has also been transmitted to goats housed with infected horses.[1] Laboratory animals are also susceptible, including mice, hamsters, guinea pigs, rabbits, and monkeys.[1] Cattle, swine, and chickens appear to be resistant, even after experimental injection.[1,59,60] Pigeons were infected experimentally.[1] A review of experiments on glanders in animals led Loeffler to suggest that the field mouse, donkey, mule, horse, goat, cat, and guinea pig were more susceptible to glanders infection and clinical disease than humans.[1] Among other susceptible host species, the rabbit and dog appeared to be less susceptible to disease than humans. Recent reports have described the use of invertebrate species including wax moth larvae, cockroaches, and nematodes as viable experimental models of glanders that are primarily useful for identifying virulence factors.[61–63]

Pathogenesis

Overview of Pathogenesis

Although glanders has been largely eradicated in humans, and for the most part in animal populations, *B mallei* is considered a significant potential biothreat agent.[64] Both the acute and chronic forms of glanders were described in detail long before effective treatments were available and when the disease was still prevalent. In the 1906 review of 156 chronic human glanders cases, Robins stated that distinguishing between chronic and acute disease was difficult because chronic disease was often interrupted with

acute symptoms and acute onset disease may run a chronic course.[3] For convenience purposes he defined chronic cases as those lasting longer than 6 months. Most historical literature attempting to differentiate the two classifies a more fulminant and rapidly fatal clinical course (within 2 to 4 weeks) as an acute form of glanders. An acute course is found more often with untreated acute pneumonic and frank septicemic infection, whether primary or recurrent.[5,47,65] Chronic infections are most common in horses where they comprise the majority of cases, whereas acute disease is more common in humans and donkeys.[7]

B mallei most often enters the human body through abrasions or openings in the skin, particularly where occupationally exposed on the hands and forearms, face, and neck. An abrasion is not always present, however, at least grossly. Normal, intact skin resists penetration of the organism; however, in several human infections, the affected persons insisted no wound or penetration occurred during the likely exposure interval. Thus, a patient history in which there is no recollection of exposure to horses or of abrasion should not preclude glanders as a differential diagnosis. Organisms may also enter through oral, nasal, and ocular mucous membranes, as well as via inhalation. The latter has occurred in several laboratory-acquired infections; however, at least one laboratory-acquired case most likely occurred through cutaneous exposure. When present, the most characteristic feature of the disease is glanders nodes—small papular to egg-sized abscesses—that are very slow to heal if they open.

The incubation period for glanders is variable, ranging from less than a day to several weeks. Cutaneous and mucous membrane exposure generally leads to symptoms in 3 to 5 days; without direct inoculation of the organism, however, the duration may be longer.[3] Inhalational exposure may incur a slightly longer range of about 7 to 21 days.[3,5]

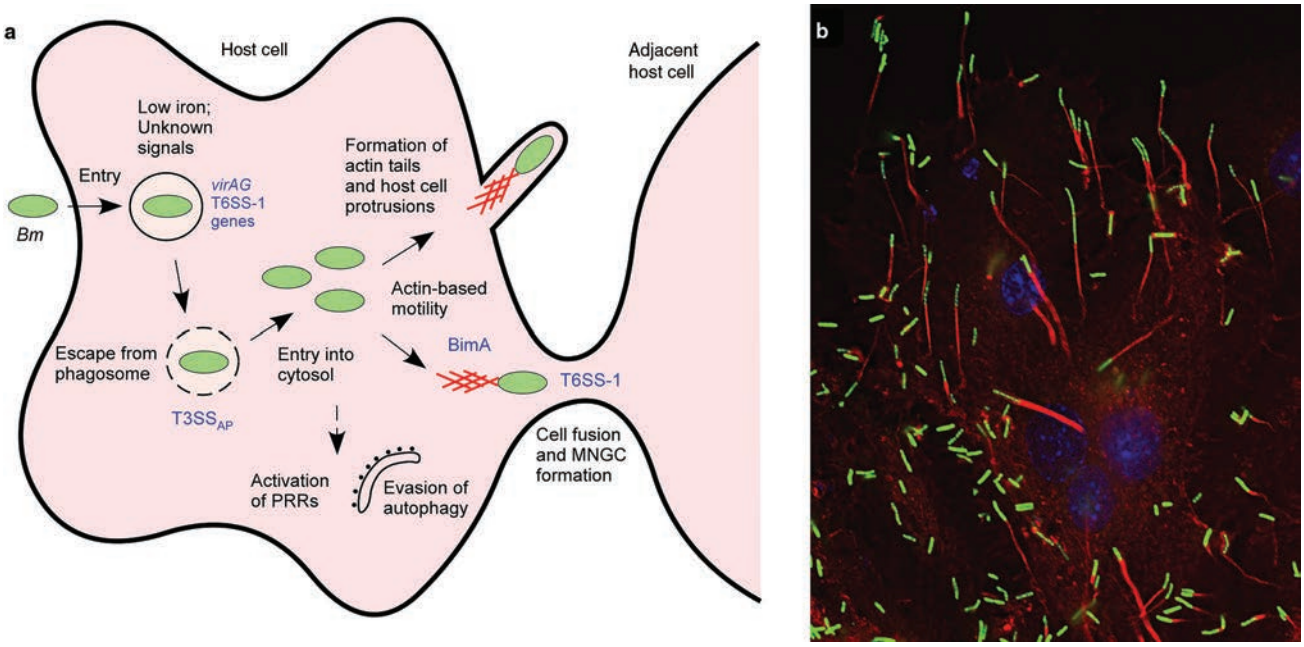


Figure 8-2. Interactions of *Burkholderia mallei* with host cells. (**a**) Proposed model of the intracellular lifestyle of *B mallei* in phagocytic cells. Following entry in host cells, *B mallei* rapidly escapes from the phagosome and enters into the cytosol where it can grow, polymerize host cell actin (*red*), spread cell to cell, and induce host cell fusion resulting in the formation of multinucleated giant cells. *B mallei* interacts with various pattern recognition receptors and evades host cell autophagy. Genes, proteins, or systems that are known to be important at various points are indicated in *blue text*. VirAG senses a signal within the phagosome that activates T6SS-1 gene expression; $T3SS_{AP}$ is required for escape from the phagosome; BimA is necessary for actin-based motility and actin tail formation; T6SS-1 is critical for multinucleated giant cell formation. (**b**) Fluorescent micrograph of *B mallei* infected RAW 264.7 murine macrophages. *B mallei* was added to RAW 264.7 cells at a multiplicity of infection of 10; at 12 hours postinfection cells were fixed and stained for actin and nuclei. *B mallei* expressing green fluorescent protein is shown in *green*; actin stained with Alexa Fluor^{568} phalloidin is shown in *red*; nuclei stained with DRAQ5 are shown in *blue*. MNGC: multinucleated giant cell
Photograph: Courtesy of Mary N Burtnick, University of South Alabama, Mobile, Alabama.

Intracellular Lifestyle

B mallei is a host-adapted deletion derivative of *B pseudomallei* and is genetically more uniform and less diverse than the latter.[66,67] The severity, clinical course, and frequent chronicity of glanders are likely related to the capacity of *B mallei* to survive and persist within host cells and thus evade destruction by the immune system. As an intracellular pathogen, survival of *B mallei* involves binding to and invading eukaryotic cells, successfully escaping phagosomal compartments, and growing in the cytosol. Relatively little is known concerning how the organism binds to cells or the surface receptor involved. *B mallei* adheres poorly to and does not invade A549 and LA-4 respiratory epithelial cell lines, but readily invades phagocytic cell lines such as J774.2 and RAW 264.7 murine macrophages.[68–70] As shown in Figure 8-2 and as discussed below, *B mallei* uses a type III secretion system to escape from the phagosome into the cytosol, where it can multiply and use actin-based motility to move about the cell.[68,69,71,72] In addition, *B mallei* can induce cell-to-cell fusion resulting in the formation of multinucleated giant cells (MNGCs), which are thought to provide a protective niche and metabolic resources for the bacteria, but little is known about the bacterial or host factors that are required for MNGC formation. Recent evidence points to expression of a type VI secretion system as a requirement for efficient intra- and intercellular spread and host cell fusion.[73,74] *B mallei* can also evade innate host immune responses when present extracellularly due in part to its surface structures, which will be described below. However, little is known about the specific molecular mechanisms of *B mallei* virulence, and more study is urgently needed.

Animal Models

Research on pathogenicity requires the availability of relevant, well-characterized animal models. Various animal models of glanders have been reported for use in studies on pathogenesis and countermeasure evaluation, including guinea pigs, mice, hamsters, nonhuman primates (NHPs), and several invertebrates. Major models will be described and are summarized in Table 8-1; several recent reviews are available that provide further details of the development of in vivo models for glanders.[44,75,76]

Guinea Pigs and Hamsters

Guinea pigs and hamsters are the laboratory animals exhibiting the greatest susceptibility to *B mallei*, and guinea pigs were initially used most extensively. However, these animals varied in their individual susceptibility to infection. Syrian hamsters proved to be uniformly susceptible to infection and have been used more extensively in recent studies on *B mallei*.[44,60,75,77] In 1999, Fritz et al characterized disease in these animals bacteriologically and pathologically to include gross, histological, immunologic, and electron microscopic pathology.[78] The hamster was shown to be much more susceptible to *B mallei* than is presumed for humans, with an LD_{50} of less than 10 colony-forming units (cfu) by the intraperitoneal (IP) route, with mortality/morbidity monitored for 5 days.[77,78] Nevertheless, the course of disease and extensive development of glanders-associated pathology in a broad range of organs, especially in reticuloendothelial organs (such as spleen, lymph nodes, liver, and bone marrow) but also in other organs such as lung and brain, are similar to those in humans. The changes observed consist of infiltrates with an equal mixture of macrophage and polymorphonuclear leukocyte inflammatory cells; these can become organized into discrete, often bacteria-filled, nodules referred to pyogranulomas. More extensive information on the pathology of glanders in these susceptible species is described elsewhere.[44]

Mice

Mice vary by strain in their susceptibility to *B mallei*, a finding that mirrors that observed in studies with *B pseudomallei*.[79–81] However, mice are all considered to be moderately resistant to infection, similar to humans. Depending on the strain, route, and dose of infection selected, they have been used to model a range of disease manifestations and states ranging from latent to acute or chronic. Since genetic constructs and reagents specific to mice are widely available, they are common tools for studies on pathogenesis and protection. In most studies, the C57Bl/6 strain was more resistant than BALB/c strain mice by the pulmonary route.[44,76,82] Studies by Goodyear et al have shown that when *B mallei* was administered intranasally to C57Bl/6 mice, high dose inocula (5,000 cfu) resulted in acute infections that were lethal within 3 to 4 days, whereas low dose inocula (500 cfu) resulted in chronic infections.[82] In recent as well as previous studies, the relatively more sensitive BALB/c strain has been used most often.[44,76,83,84] In acute infection studies with BALB/c mice, a lethal IP dose often results in splenomegaly with multiple splenic white foci consisting of pyogranulomatous inflammation. This pathology occurs also in other reticuloendothelial organs, as typically described for hamsters; but mice usually do not exhibit this pathology in other organs, such as the lung, as occurs later in infection of hamsters.[44,83]

TABLE 8-1

EXPERIMENTAL ANIMAL MODELS OF GLANDERS

Model	Route of Infection	Features
Horses	IT	Physiologically relevant model that mimics the natural development of chronic glanders in its reservoir host. Horses typically exhibit a long disease course with periods of improvement and relapse.
Rhesus monkeys	SC and aerosol	Development of SC lesions that healed after 3 weeks. No evidence of chronic or acute infection. Evidence of potential nonlethal chronic infection after aerosol exposure.
African green monkey	Aerosol	Development of acute glanders resembling the human infection.
Syrian golden hamsters (*Mesocricetus auratus*)	IP	Extremely susceptible infection model (LD_{50} <10 cfu) with development of acute infection resulting in a rapid disease course. Bacteria are transported to the mediastinal lymph nodes and seeded to other tissues, forming lesions in the spleen as early as 1 day postinoculation and death occurring around 6 days later.
Guinea pigs	SC and IP	Development of acute glanders. Bacteria are transported to inguinal and axillary lymph nodes (SC), or mediastinal and mesenteric lymph nodes (IP), at early time points postinoculation.
BALB/c mice	Aerosol, IN, and IP	Good model for acute and potentially chronic glanders. Commonly used model due to low cost, susceptibility to *B mallei* infection, and a well-documented disease pathology. Mice are more resistant to *B mallei* when delivered IP.
C57Bl/6 mice	Aerosol, IN, and IP	More resistant to *B mallei* infection than BALB/c mice by pulmonary routes. High doses delivered IN resulted in acute disease, whereas low doses produced chronic infections.
Wax moth larvae (*Galleria mellonella*)	Injection into hemocoel	Used to screen putative virulence mutants; between 3 to 200 cfu of wild type *B mallei* leads to >90% killing within 6 days.
Madagascar hissing cockroaches	Injection into dorsal abdomen	Used to screen putative virulence mutants; highly susceptible infection model (LD_{50} <10 cfu) resulting in death within 5 days.
Nematodes (*Caenorhabditis elegans*)	Feeding	Used to screen putative virulence mutants; limited sensitivity resulting from high infectious dose.

cfu: colony forming unit
IN: intranasal
IP: intraperitoneal
IT: intratracheal
LD: lethal dose
SC: subcutaneous

Aerosol exposure to a lethal dose of *B mallei* produces gross and microscopic pathologic changes similar to those after IP injection, except that there is more lung involvement, that is, focal inflammation and necrosis early in infection develop into extensive consolidation with chronic inflammatory cell infiltration (David L Fritz, David DeShazer, David M Waag, USAMRIID, Fort Detrick, MD, unpublished data, 2012).[84] It has been observed that aerosol-exposed BALB/c mice develop acute inflammation of the nasal passages, which later extends to the nasal sinuses and ultimately into the brain, especially the olfactory lobe (David M Waag, USAMRIID, Fort Detrick, MD, unpublished data, 2013).[84] Unlike humans, mice are obligate nose breathers, an anatomical difference that may explain these findings. Recent models using an intratracheal (IT) route of infection address this potentially confounding issue.[85] However, the brain infection in mice might serve as a potential protected site for bacterial survival and provide a model for studying recrudescence of disease.

Acute infection virulence data obtained with murine models often used laboratory strains such as the type strain *B mallei* ATCC 23344 (aka China 7) or the most

recent human clinical isolate of *B mallei*, referred to as the FMH 23344 strain of *B mallei* ATCC 23344.[56] The 50% lethal doses (LD_{50}s) for BALB/c mice have varied by route, and the mice were more susceptible by pulmonary routes than by the IP route. The range of LD_{50}s, as determined 10 to 35 days postchallenge (most 21 or 28 days), is exemplified as follows for BALB/c mice: IP (7 x 10^5 cfu), intranasal (IN; 680 cfu), IT (818 cfu), whole-body aerosol (913 cfu), and nose-only aerosol (1,859 cfu).[76,77,83–88] Recently, the virulence of *B mallei* strain FMH 23344 was reevaluated in C57Bl/6 and BALB/c mice challenged by the whole-body aerosol route. As expected, C57Bl/6 mice were significantly more resistant than BALB/c mice; the respective LD_{50}s (after 21 days) were 7,665 cfu and 395 cfu, respectively (David M Waag, USAMRIID, Fort Detrick, MD, unpublished data, 2013).

No well-defined murine models for long-term chronic or latent glanders exist. However, *B mallei* clearly establishes protracted persistent infections in mice as it does in horses and humans, as shown especially in BALB/c mice. In recent aerosol and IP challenge studies, spleen cultures revealed the presence of long-term residual infection with *B mallei* in surviving mice. Such infections were more consistently detected in BALB/c than in C57Bl/6 survivors, that is, BALB/c mice surviving greater than or equal to 60 days after aerosol challenge with the FMH derivative of strain *B mallei* ATCC 23344 had positive spleens; whereas C57Bl/6 survivors had usually cleared the infection (Christopher K Cote and David M Waag, USAMRIID, Fort Detrick, MD, unpublished data, 2012). Fritz et al conducted a natural history study of *B mallei* ATCC 23344 in mice infected lethally and sublethally by the IP route.[83] Pyogranulomatous inflammation was observed histologically in multiple reticuloendothelial organs, and the incidence and severity of the changes did not decrease in the sublethally infected mice. Barnes et al treated mice that had been infected by aerosol with *B mallei* with a postexposure prophylactic regimen of trimethoprim-sulfamethoxazole that effectively prevented acute infection; two-thirds of the mice survived to study end at day 74.[89] However, the treatment did not eradicate the bacteria and a clinical relapse of infection occurred by day 30 postchallenge. *B mallei* was detected in the organs of all surviving mice. Similar data have been reported, and the results overall suggest that BALB/c mice could serve as a useful model for both acute and chronic glanders in a relatively resistant host such as humans.[89,90] Further research to develop defined murine models for persistent forms of *B mallei* infection are needed to identify the factors involved in the evolution of infection.

Nonhuman Primates

Although NHPs are phylogenetically the closest animal to humans, few studies have described their use as laboratory models. In one study, rhesus monkeys were given different doses of a virulent strain by the subcutaneous route, and the monkey receiving the highest dose developed a cutaneous abscess that resolved completely after 3 weeks.[44,60,76] Russian investigators reported studies with baboons but few details are available.[44] Different species of NHPs appear to vary significantly in susceptibility but overall there is a lack of substantial data characterizing experimental infection in these animals. The challenge is to develop a model(s) that best mimics the human acute and chronic responses to *B mallei*. Recent studies have shown that rhesus macaques are resistant to acute infection (John J Yeager and M Louise Pitt, USAMRIID, Fort Detrick, MD, unpublished data, 2012); however, Yingst et al showed that rhesus monkeys given subclinical doses of *B mallei* exhibited clinical presentations and pathological lesions that correlated well with those described for human cases of glanders.[91] They suggested that the rhesus macaque is a potentially viable model for human disease albeit not acute lethal illness. A longer-term study is being done to include LD_{50} determinations with extensive clinical, pathological, and laboratory evaluations to further analyze three NHPs as models of human glanders (Patricia L Worsham, David M Waag, and Taylor B Chance, USAMRIID, Fort Detrick, MD, unpublished data, 2013). African Green monkeys (*Chlorocebus aethiops*) were much more sensitive to infection than cynomolgus macaques or rhesus macaques and appear to be a potential model for acute infection (Patricia L Worsham, David M Waag, and Taylor B Chance, USAMRIID, Fort Detrick, MD, unpublished data, 2013).

Invertebrate Models

Several nonmammalian surrogate models have recently been used to study virulence mechanisms and host-pathogen interactions of the human pathogenic species of *Burkholderia,* including insect larvae, cockroaches, a phagocytic amoeba, and soil-dwelling nematode. For *B mallei*, models using wax moth larvae *(Galleria mellonella*) and the Madagascar hissing (MH) cockroach have been reported.[62,63] Insect models are useful mammalian surrogates for several reasons. Significant similarities exist between the innate immune systems. Both hosts harbor Toll receptors (insects) or Toll-like receptors (TLRs, mammals) that recognize pathogen markers and produce protective responses such as antimicrobial peptides; also insects possess

phagocytic hemocytes that can take up and kill microbes, in a manner similar to that of neutrophils. Other advantages of using insects as models involve ready availability, reduced costs and facile housing/maintenance, and exemption from regulatory oversight and expense. *B mallei* was shown to be as virulent for the larvae as it was for hamsters and mice, whereas *Burkholderia* that are nonpathogenic in mammals were not pathogenic for the insects. Notably, in tests with mutants of *B mallei* harboring known virulence-associated gene defects (eg, in capsule production or in the type three secretion system [$T3SS]_{AP}$), lethality for wax moth larvae corresponded to the extent of reduced virulence of the mutants in hamsters and mice.[63] Fisher et al found the MH cockroach to be easier to handle than wax moth larvae and their ability to grow at 37°C made them more amenable than other insects to mutant analysis with *B mallei*, a mammalian host-adapted pathogen.[62] Thus, MH cockroaches appear to be a valid surrogate and alternative to mammals as a model for virulence mechanisms of *B mallei* important in host interactions.

Virulence Mechanisms

Surface Polysaccharides. Since *B mallei* appears to be genetically derived from the environmental saprophyte *B pseudomallei*, it shares many virulence factors with the latter.[75,92] However, some of the *B pseudomallei* factors required for its independent lifestyle appear to have been lost in *B mallei* as a consequence to its adaptation to the equine hosts. In addition, differences in the presence or role of some virulence factors in *B mallei* compared to *B pseudomallei* have been described (possibly related to the increased presence in *B mallei* of insertion sequence elements and genetic rearrangements), as will be illustrated. The factors and activities identified as being essential for *B mallei* virulence and host persistence include the following:

- a capsular polysaccharide (CPS);
- lipopolysaccharide (LPS);
- animal pathogen-like T3SS ($T3SS_{AP}$);
- the cluster 1 T6SS (T6SS-1); and
- the VirAG two-component regulatory system.[38,72,74,77,93]

Other putative virulence factors, including various autotransporter proteins, adhesins, quorum sensing, and iron-binding compounds, have been identified but their roles in virulence are unconfirmed (as described below).

Two major polysaccharide (PS) antigens that are present on the surface of *B mallei*, a CPS and LPS, play important roles in the pathogenesis of glanders and in host responses to the infection. The presence of a CPS on the surface of *B mallei* was shown by immunoelectron microscopy.[77] The structure of the CPS antigen has recently been characterized and shown to be identical to the CPS expressed by *B pseudomallei*, which is a homopolymer of -3)-2-*O*-acetyl-6-deoxy-ß-D-manno-heptopyranose-(1-.[77,94] Consistent with this finding, anti-CPS monoclonal antibodies (mAbs) that have been characterized recognize the CPS of both pathogens.[95–97] The surface-expressed nature of the LPS is evidenced by its availability to the host immune system and its ability to activate TLR4 complexes.[97–99] The structure of *B mallei* O-polysaccharide (OPS) has been determined to be a repeating unit of -3)-β-D-glucopyranose-(1,3)-6-deoxy-α-L-talopyranose-(1- where the talose residues contain 2-*O*-methyl or 2-*O*-acetyl side groups.[93,100] In comparison to *B pseudomallei*, *B mallei* OPS lacks 4-*O*-acetyl modifications on the talose residues.[93,100,101] This structural difference explains why mAbs specific for either *B mallei* LPS or *B pseudomallei* can be isolated.[101,102] The virulence roles of these PS antigens were demonstrated by the construction of mutant strains lacking either CPS or OPS, which proved to be avirulent in animal models.[77,93,103] The precise functions of CPS and LPS in pathogenesis are not fully characterized; however, the CPS may contribute to survival in serum by inhibiting complement deposition, opsonization, and phagocytosis, as well as possibly conferring resistance to the harsh environment of the phagosome until the bacteria are able to escape.[75] OPS is known to be critical for serum resistance since *B mallei* strains lacking OPS moieties are rapidly killed by 30% normal human serum.[93] mAbs to both *B mallei* CPS and LPS have been identified that are either bactericidal for the organism or have strong opsonic activities.[97] The roles of these two PS moieties in pathogenesis were further confirmed by demonstrating that passive administration of LPS- or CPS-specific mAbs effectively protected mice against lethal pulmonary challenge.[97,104]

Secretion Systems and Secreted Proteins. *B mallei*, a highly successful facultative intracellular pathogen that can survive in many eukaryotic cell lines, possesses a variety of mechanisms to adapt to and alter the host environment.[68,69,71] The organism harbors an array of specialized secretory systems that are essential to this process. Little is known about these systems or the specific roles of their components, although a number of the genes identified appear to be homologous to the more extensively studied species *B pseudomallei*. *B mallei*-specific studies have focused primarily on characterization of $T3SS_{AP}$ and T6SS-1.[69,72–75,105,106] Genes encoding other secretion systems, including the type II and type V systems, are also present in *B mallei*.[75] The effector proteins delivered by these systems are

predicted to disable or modulate critical host proteins and pathways involved in cell signaling, cytoskeleton and ubiquitin function, and cell death pathways, thus facilitating pathogen survival and propagation in the host.[70,75,107–109] In addition, both *B mallei* and *B pseudomallei* exhibit actin-based motility and the distinct ability to induce formation of MNGCs in tissue culture models, potential mechanisms that allow the pathogen to spread in the host via direct cell-to-cell passage (Figure 8-2).[73,75,110]

After *B mallei* is phagocytosed by the host cell, it escapes from the endocytic vacuoles into the host cell cytoplasm where it uses actin-based motility to spread intra- and intercellularly. Virulence-associated $T3SS_{AP}$ and T6SS-1, as well as other secreted proteins, appear to be essential for these various functions.[68,69,71–73,75,103,111,112] Many gram-negative bacterial pathogens harbor such secretion systems and use them to synchronize the secretion and delivery of effector proteins directly into target host cells via needle-like injection apparatuses. *B mallei*, $T3SS_{AP}$ is required for virulence in animal models of infection, as well as for phagosomal escape and survival in macrophage tissue culture models of infection.[69,72,75] Once free in the cytosol, the microbe activates processes for evading host cell killing and for polymerization of host cell actin.[75] BimA, a type V secreted (T5S) protein, plays a major role in facilitating actin-based motility in *B mallei*.[113,114]

Although the $T3SS_{AP}$ is important for phagosomal escape and survival within host cells, the exact roles of specific effector proteins delivered by this system have yet to be clearly identified. Many proteins are predicted to be part of the *B mallei* T3SSs by in silico annotation; some have been partially characterized, and the potential roles of a few in virulence have been studied.[75,92] These proteins include BopA and BopE; Bip B, C, and D; and BapB, as described in detail in previously published reports.[70,75,92] In *B mallei* a mutation in the T3SS-encoded BopA effector protein resulted in a slower growth rate in macrophages and an apparent reduced ability to escape the cells. This mutation also attenuated infection by *B mallei* in BALB/c mice, suggesting BopA may contribute to survival within—and possibly escape from—host alveolar macrophages.[70,115] In *B pseudomallei*, BopA appears to enhance survival by helping the microbe evade autophagy-induced phagocytic vacuole degradation.[116] Bip B, C, and D proteins appear to be structural components of the T3SS injector apparatus that are involved in contact of the tip with host cells.[75]

T6SS-1, which is important during the intracellular lifestyle of the *B mallei*, is essential for *B mallei* virulence in a hamster model of glanders.[63,74] In a RAW 264.7 macrophage model, T6SS-1 expression was shown to occur following internalization of *B mallei*, but before escape of the organism from the phagosomal environment.[73] Once in the cytosol of host cells, T6SS-1 mutants displayed defects in actin polymerization and an inability to induce MNGC formation.[73,105] T6SSs are proposed to resemble inverted bacteriophage tail-like structures involved in delivering effector molecules directly into target cells. These systems are tightly regulated at the genetic level so that they are only expressed at the appropriate time and place. Two key components of T6SS-1 are the Hcp1 and VgrG1 proteins, which are both secreted and structural components of the T6SS apparatus and are considered reliable indicators of T6SS function.[74,112,117,118] Various components of T6SS-1 are being characterized because these proteins may represent potential diagnostic, therapeutic, or vaccine targets.[73,74,105,111,112]

The VirAG two-component system is an important regulator of virulence gene expression in *B mallei* and is required for virulence in hamsters.[38] Approximately 60 genes are under the regulatory control of VirAG, including the T6SS-1 gene cluster and genes involved in actin-based intracellular motility (bimBCADE).[74] When the VirAG system is overexpressed in vitro, Hcp1 and VgrG1 are secreted into culture supernatant by T6SS-1. VirAG also controls the expression of *tssM*, which encodes a putative ubiquitin-specific protease (ie, deubiquitinase) that is expressed shortly after intracellular uptake and provides the bacteria with an enzymatic tool that can potentially regulate multiple eukaryotic cell processes.[74,108] Shanks et al characterized the expression and regulation of *B mallei* TssM, and demonstrated that it was a potent ubiquitin-specific protease.[108] Ubiquitin is a host protein that attaches to other proteins so as to direct their intracellular fate. Bacterial deubiquitinases remove the ubiquitin residues, disrupting this process and promoting bacterial evasion of host immune responses and survival. Although the TssM protease may provide *B mallei* a selective advantage within the cell during infection, a role in virulence for hamsters was not shown. Interestingly, even though *tssM* is coregulated with and physically linked to the T6SS-1 gene cluster, TssM is not secreted by either T6SS-1 or $T3SS_{AP.}$[108] More research is needed to determine the signal sensed by VirAG in vivo that ultimately results in the expression of this important regulon.

Autotransported and ATP-binding Cassette Proteins. Several autotransporter (AT) and ATP-binding cassette (ABC) transporters that may have roles in the infectious process have been described for *B mallei*. ATs are large families of outer membrane proteins in gram-negative bacteria that are secreted by the T5S pathway, and they include virulence-associated

invasins, adhesins, proteases, and actin-nucleating factors.[119,120] AT proteins have three common features: (1) an N-terminal signal sequence for periplasmic translocation, (2) a central functional domain(s), and (3) an outer membrane channel-forming C-terminus needed for surface export of the central domain.[119] Eight of the 11 AT analogs of *B pseudomallei* are shared by *B mallei* and include BimA, an AT involved in actin tail formation and actin-based motility, and BoaA, an AT with a potential role in bacterial adhesion to epithelial cells.[74,76,114,121] BimA is expressed by both *B pseudomallei* and *B mallei*, although their sequences vary. In both species BimA is required for actin-based motility and MNGC formation in infected tissue culture monolayers.[74,76,114,122] Interestingly, in *B mallei*, the bimBCADE genes were found to be dispensable for virulence in hamsters.[74]

A group of immunodominant *Burkholderia* antigens, designated Hep-Hag autotransporter (BuHA) proteins, shares structural similarities with hemagglutinins and invasins.[123] These proteins were present in 53% of a *B mallei* expression library examined and only 3% of a *B pseudomallei* library. They appear to function as surface proteins that modulate interactions of the bacterial cell with the host and environment; homologs in other bacteria have significant roles in virulence, but their possible roles in *B mallei* virulence and immune modulation require further study. Finally, several ABC protein systems with established roles in the virulence and pathogenicity of various gram-negative pathogens have been identified in the *Burkholderia*.[124,125] Although their contribution to *B mallei* pathogenicity has not been evaluated, some components (eg, the ABC transporter protein LolC) have been shown to be immunogenic and to elicit significant partial protection against both *B mallei* and *B pseudomallei*. These proteins deserve further analyses as both putative virulence factors and vaccine targets.

Quorum Sensing Systems. Quorum sensing (QS) permits bacteria to monitor their population density and modify gene transcription at critical population levels.[126] Many host-associated bacteria use small amphipathic acyl-homoserine lactone signals for QS; and Duerkop et al identified octanoyl-homoserine lactone as the predominant BmaI1 synthase-produced acyl-homoserine lactone signal and activator for the *B mallei* LuxR QS system.[126] Nevertheless, numerous animal pathogens lack such systems, yet are virulent. Using mutants in bmaI genes of the *B mallei* QS systems, Ulrich et al showed that QS was critical for virulence of *B mallei* in aerosol-exposed BALB/c mice.[127] However, using constructs with similar mutations in bmaI, Majerczyk et al recently determined that QS was not required for lethal infection of mice exposed by aerosol to *B mallei*.[128] These studies do not exclude a role for QS in glanders in other animal models or the natural equine host.

Other Potential Virulence Factors. A role for pilin/fimbriae structures in the pathogenesis of glanders is poorly defined.[75,129] For instance, type IV pili are required for virulence of *B pseudomallei*, and although they are expressed by *B mallei* in vivo and are highly immunogenic, a role for them in adherence and virulence of *B mallei* has yet to be shown. Neither active nor passive immunization with pilin or anti-pilin antibodies protected mice against subcutaneous or aerosol challenge. Its protective role in a natural host or incidentally infected human or after exposure by different means remains to be determined.[129] Several other virulence mechanisms are being investigated, and innovative techniques and combination approaches (in silico, in vitro, and in vivo) are being used to identify putative novel virulence factors with possible roles in pathogenesis of *B mallei* or in protective immunity.[67,92] One of these virulence mechanisms is the carboxy-terminal processing protease of *B mallei* and compounds potentially involved in iron acquisition, such as the siderophore malleobactin.[130–132]

Clinical Disease in Animals

B mallei naturally infects horses, donkeys, and mules, but other species have occasionally become infected.[41,45,58,133] If glanders is suspected as a differential diagnosis, local and regional animal and public health authorities must be immediately notified. The incubation period for glanders in equids ranges from a few days to many months, with most between 2 to 6 weeks. The infectious process, disease progression, and pathology in equids are similar to those in humans. Donkeys are most likely to succumb to acute disease and die in a week to 10 days.[1,4] Horses are more likely to incur a slowly progressive, chronic disease. Recurring clinical disease and even death in horses may manifest months to years after dormancy, particularly after any stress that causes a rise in temperature such as infectious disease, roundup, transport, overwork, poor diet, exercise, immunization, and even mallein testing.[1,4,134] Changes in season, from winter to spring and from summer to fall, have also been associated with recurrent disease.[4]

The primary route of infection in the natural host is oral, by chewing or contacting contaminated food and water, feeding and husbandry equipment, and by direct close contact with infected animals.[135] Tooth eruption, irregular tooth wear, coarse feeds, and bridling contribute to oral trauma, a common finding that leaves the mucosa and mucocutaneous junctions more

vulnerable to infection. Equids are also gregarious and prefer to be in close contact with at least one other. Grooming and nibbling behavior also exacerbate the potential for exposure from direct contact. Contaminated aerosols, such as those produced by snorting or coughing, may also easily find their way into the eyes, mouth, or skin abrasions of other equids in the vicinity. Tack, such as a harness, can cause skin irritation that—if contaminated—may allow easy entry of the organism. Despite the oral route of infection, significant pathology is usually seen in the airways and lungs.[40]

With early infection or resurgence, constitutional signs are often the first to manifest. These signs may include thirst, fever (low grade to high), shivering, head drooping, tachycardia, tachypnea, weight loss, rough hair coat, indolence, prostration, and a reluctance to move.[136] Swelling of the limbs and joints may be seen. The lungs, mucosa of the respiratory tract, and lymphatic system are most frequently involved wherever the infection originates. Horses experimentally infected by cutaneous flank injection of infectious material developed a respiratory tract infection within a few weeks.[1] In some cases (or at various stages of disease) the lungs may appear to be the only organ involved. Regional or diffuse pneumonia and pleuritis are common. The lungs and upper respiratory tract are also the organs and tissues that show the oldest signs of chronic disease. Lung pathology is typically more marked and extensive in donkeys than in horses.

The nasal form of glanders classically described in equids is a somewhat local infection of the nasal cavity at least characterized by yellowish-green unilateral or bilateral nasal discharge, with or without nodules or ulcers on the nasal mucosa. Regional lymphadenopathy and lymphangitis most often accompany nasal signs. Laryngeal, tracheal, and lower respiratory tract pathology is often present, however, even if microscopically supporting the concept that a local infection is more likely just early infection, or rare. Nasal signs are common with recurrence of chronic infection. Although the nasal form has been associated with equids, similar pathology has been described in humans.[3,54]

With clinical expression of upper respiratory infection, a highly infectious, sticky, yellow-gray to greenish viscid unilateral or bilateral nasal exudate is produced. The glottis may be edematous and nasal discharge may be so thick as to obstruct nasal passages. The margins of the external nares are often swollen and crusted. The exudate may be periodically blood-tinged. The muzzle and distal forelimbs may be covered with this exudate; the latter is due to wiping the nose. The nasal mucosa may be nodular and ulcerous with ulcers often rapidly spreading. Ulcers may be deep and coalesce forming larger ulcers. Mucosal abscesses of the septum and nasal conchae may have swollen edges and display small yellow and gray nodules. These abscesses may invade the turbinates and cartilaginous structures, leading to perforation and erosion of the nasal septum. Particularly where the larger ulcers heal, white stellate or radial scars are left on the mucosa. These scars may be seen with an endoscopy, and they are near-hallmark signs of prior infection. Visible or palpable regional lymphadenopathy (particularly submandibular) and lymphangitis are present.

The equid will frequently snort to clear nasal passages, effectively showering the immediate area with the infectious exudate. The animal may cough, or a cough may be easily elicited by placing pressure on the throat over the larynx when there is laryngeal involvement. The air-exchange produced by a cough may exacerbate nasal discharge as equids breathe through their nose and not their mouths. Dyspnea, particularly inspiratory, may result from swelling in the nasal cavity or larynx. Expiratory dyspnea is also not uncommon, particularly with chronic involvement of the upper and lower respiratory tract.[55] Auscultation and diagnostic imaging findings may support localized or diffuse lung disease and pleurisy. Clinical signs may be mild and transient, or severe and progressive. Death may occur within a few days.

At necropsy, glanders nodes will likely be found in the lungs, even if incidentally. Their consistency may be caseous to calcified depending on lesion age. These nodes may be of any size and occur as just a few or as hundreds in a diffuse miliary pattern. Pleuritis may also be found at necropsy. The microorganism is relatively abundant in the affected tissues. Animals may die within 3 to 4 weeks from bronchopneumonia and septicemia.

The progression of cutaneous and mucous membrane infection in the equid is similar to infections in humans. An entry wound may not be found. Lymphatic involvement may be more visible, however. Subsequent to cutaneous or mucosal infection, regional lymphangitis develops within 7 to 10 days. Typically, the lymphatics undergo a visible or palpable "string of pearls" stage within 10 days and then turn to more solid, fingerlike cords that can be traced to regional lymph nodes. Nodules along the lymphatic vessels may erupt, exuding gelatinous pus. Lymph nodes may be enlarged and indurated, and less frequently they may rupture and suppurate. With disease progression, more eruptions, enlargement of eruptions, and coalescence of lesions are expected. As a rule these are very slow to heal. Thick crusts of wound secretions, hair, bedding, and dirt may mat around the lesions. With ocular involvement, photophobia, excessive lacrimation, mucopurulent ocular discharge, conjunctivitis, and apparent partial

blindness may occur, and this may result in behavioral changes such as avoidance or fear. With disseminated disease, cutaneous and mucous membrane lesions may appear anywhere, particularly the respiratory tract, as previously mentioned, and the limbs. The hind limb is more commonly affected than the forelimb.[42,48]

Acute septicemia may occur at any stage of infection. A septicemic course is typically progressive with signs leading to multiple organ failure including watery diarrhea, colic, marked dyspnea, prostration, cardiovascular collapse, and death. Donkeys are particularly susceptible to *B mallei* septicemia; this form manifests in most of those naturally and experimentally infected. Disseminated disease in horses is typically more protracted, however. Clinical signs vary widely and may include any of those previously mentioned. Horses may be asymptomatic. They may appear slightly thin, unthrifty, or have an occasional or persistent nasal discharge. There may be a transient mild to moderate fever. Mucous membrane and cutaneous lesions as well as lymphadenopathy and lymphangitis may also be transient or chronic. Visceral abscess is common, and the spleen and the liver are frequently involved. Intact males may have orchitis, which may not be evident without a reproductive examination.[40,137] Remission is unlikely with disseminated disease particularly if it involves visceral organs.

In the event an equid presents with clinical or necropsy signs consistent with glanders, the premises should be immediately quarantined and local and regional animal health authorities should be notified. Treatment should not be attempted. Although a clinical prognosis for various forms of glanders infection may be surmised, it is less relevant now because of the global interest in eradicating (by test-and-slaughter) the disease.

Chronically infected horses may display cycles of worsening disease followed by apparent recovery where few symptoms are displayed. Clinical signs include intermittent cough, lethargy, and lesions in the nasal region, lungs, and skin, just as with acute disease.[136] Lungs may develop lesions similar to tubercules. Nodules may appear in the submucosa of the nasal cavity, particularly the nasal septum and turbinates. Nodules found in the liver and spleen may be up to 1 cm in diameter and have a purulent center surrounded by epithelioid and giant cells.[138] Attempts to isolate *B mallei* from chronically infected animals are usually unsuccessful. Thrombosis can be found in the large venous vessels of nasal mucous membranes.[25] Nodules in the skin along lymphatics may be seen in chronically infected animals as they thicken. Nodules may ulcerate and rupture, spewing a thick exudate that may be a source of infection.

Clinical Disease in Humans

Even during its peak near the turn of the 20th century, human glanders was uncommon but well documented. The clinical course of glanders is based on reports of hundreds of cases published before antibiotics were developed and from a small series of cases that occurred in the United States since the discovery of sulfonamides. The earlier reports describe a nearly always fatal disease of short (a few days to weeks) to long (months to years) duration that was usually acquired from close contact with infected equids. The most recent cases were laboratory acquired, and all patients survived.

Glanders manifestations can vary. At least five forms of infection have been described, including localized, pulmonary, septicemic, disseminated, and the aforementioned chronic infection, but none is exclusive. The most important distinction is whether the infection is localized, which is unusual except early in the infectious process. The variety of forms is largely explained by route of infection, regional lymphatic inflammation and drainage, and loci of dissemination and embolism via hematogenous or lymphatic spread. With disease progression and chronicity, all forms may manifest.

Localized infections are regionally confined and typically characterized by pus-forming nodules and abscesses that ulcerate and drain for long periods of time. Lymphangitis or regional lymphadenopathy may develop in the lymphatic vessels that drain the entry or infection site. Increased mucus production from affected ocular, nasal, and respiratory mucosa is often present. Localized infections typically disseminate, leading to pulmonary, septicemic, or disseminated infection.

Constitutional signs and symptoms typically occur early in the disease and some may persist through treatment. These signs and symptoms may be severe, leaving the patient extremely prostrate. Common signs and symptoms include fever or low grade fever in the afternoon to evening, chills with or without rigors, severe headache, malaise, generalized myalgias (particularly of the limbs, joints, neck, and back), dizziness, nausea, vomiting, diarrhea, tachypnea, diaphoresis (includes night sweats), altered mental status, and fatigue. Other nonspecific signs may be tender lymph nodes, sore throat, chest pain, blurred vision, splenomegaly, abdominal pain, photophobia, and marked lacrimation. Any or many of these signs may be present. Following constitutional signs, clinical courses are discussed in greater detail as they are associated with route of entry and disease spread.

Cutaneous manifestations include multiple papular or pustular lesions that may erupt anywhere on the body. Cutaneous or mucosal infections may spread,

leading to disseminated infections. Dissemination to internal organs produces abscesses in virtually any organ, most commonly the spleen, liver, and lungs. Disseminated infections are associated with septic shock and high mortality, yet they may also produce a more chronic, indolent course of infection.

With cutaneous entry through an abrasion, an inflammatory response of varying degrees (virulence dependent) occurs with accompanying pain and swelling. A glanders node may appear usually as a single blister, gradually developing into an ulcer that may be hemorrhagic.[7,55] Localized infection develops at the entry site with a mucopurulent discharge. Inflammation may extend along regional lymphatics and cause lymphangitis perhaps with numerous foci of suppuration along their course. This irritation is caused by endotoxins present in some *B mallei* strains affecting the smooth muscle of the lymphatics. Lymphatic vessels may be easily palpable as firm, ropy cords. Regional lymph nodes become involved and similarly inflamed. Infection may remain localized, but more often spreads, particularly without adequate treatment. Further spread occurs via the lymphatics and through hematogenous dissemination as thrombi and emboli are formed. Local endothelial tissue inflammation and suppuration can occur at any place along the route of spread, producing abscesses that may drain through the skin. Superficially, these abscesses may appear as papules or diffuse abscesses in inflamed skin, or larger (egg-sized) swellings deeper in the subcutaneous tissue and superficial musculature. Published case descriptions have described glanders nodes anywhere including the face, neck, shoulders, lumbosacral region, arms, and legs.[7,55] When the legs are affected, glanderous nodes occur more often on the medial aspect than the lateral. At first these glanderous nodes may be hard and painful, but eventually they ulcerate and slough. These nodes may exude relatively tenacious pus that varies in consistency from mucopurulent to gelatinous to oily, depending somewhat on chronicity. The nodes heal slowly and recur without adequate treatment. At any time the patient may become acutely ill and septicemic. Other organs and tissues may also be showered with infectious emboli.

The infectious process through the oral, nasal, or ocular mucus membrane is similar to the cutaneous process. Weakened or abraded membranes are more vulnerable to entry than are intact membranes. Potential entry may be associated with contaminated hands, fingers, objects, and aerosols contacting the eye, nose, and mouth. A localized infection typically follows. Within 1 to 5 days the affected membranes become injected, swell, and weep a serosanguinous to mucopurulent discharge. Papular and ulcerative lesions similar in character to those in the skin may appear. Single or multiple oral blisters and sores may also appear. Hyperemia may be diffuse (entire pharynx, conjunctiva, etc) or localized. With ocular involvement, excessive lacrimation and photophobia are common. With nasal involvement, the nose may become greatly swollen and inflamed and copious nasal discharge may occur. Infection may invade the nasal septum and bony tissues, causing fistulae and tissue destruction. The entire face can become swollen, and regional lymph glands may inflame and suppurate. Infection may also extend lower in the respiratory tract resulting in tracheitis and bronchitis, which can be accompanied by cough and the production of mucopurulent sputum. If mucous membrane involvement is extensive, constitutional signs are also usually severe including high fever, severe headache, fatigue, prostration, earache, and various neurologic signs.

Infection of the respiratory tract may be anticipated after aerosol exposure or secondarily as a consequence of disseminated infection. A pulmonary infection typically produces pneumonia, pulmonary abscess, pleuritis, and pleural effusion, with associated signs and symptoms such as cough, dyspnea, chest pain, and mucopurulent sputum. Nasal exudate and cervical lymphadenopathy may also be present if the upper respiratory tract is involved. Nonspecific signs and symptoms often accompany respiratory infections, such as fatigue, fever, chills, headache, myalgias, and gastrointestinal signs. Pulmonary abscess and pleuritis are common sequelae. Symptoms including tender cervical lymph nodes, fatigue, lymphangitis, sore throat, pleuritic chest pain, cough, fever (often exceeding 102°F), chills, tachypnea, dyspnea, and mucopurulent discharge may take 2 to 3 weeks to develop. Nonspecific signs are also usually present including night sweats, rigors, myalgia, severe headache, tachycardia, nausea, weight loss, dizziness, and mucosal eruptions. Some of the latter symptoms may indicate disseminated infection. Imaging studies may show diffuse or localized infiltration depending on the stage of infection. Miliary to necrotizing nodules or a localized (lobar to bilateral) bronchopneumonia are other potential radiographic signs. Developing abscesses may be well circumscribed and circular, later becoming cavitated with evidence of central necrosis. Pleural irritation may also be visible on imaging studies. Acute bronchopulmonic or pneumonic disease untreated tends to have a rapid onset of symptoms and was once said to be almost uniformly fatal within 10 to 30 days.[5] Most laboratory-acquired infections have resulted from inhalational exposure resulting in pulmonary infection.

Clinical features of eight laboratory-acquired infections from Camp (Fort) Detrick are summarized in Table 8-2. These infections include the six-case series

published by Howe and Miller in 1945, a previously unpublished case that occurred in 1953, and the 2000 case first presented by the Centers for Disease Control and Prevention.[11] The most common symptoms experienced by at least four of the eight include—in order of most common occurrence—afternoon to evening low-grade fever, malaise, fatigue, headache, myalgias including backache, lymphadenopathy, and chest pain (Table 8-2). Shaded elements in the table represent the first signs and symptoms according to the medical records of the first seven patients, and according to the published case description of the eighth patient. An important clinical feature that is not reflected in the table is that at least half of the patients not only "felt better" but also were clinically better for a time after the first wave of disease symptoms. This period lasted from a few days for patient 7, to 2 months for patient 2. Inhalation is suspected as the route of exposure for the first seven patients, whereas percutaneous exposure probably led to the eighth case.

Septicemic glanders results from the seeding of *B mallei* into the bloodstream, whether as a primary event, secondary to a local or pulmonary infection, or as a relapse to chronic or latent infection. Septicemia may be passing and lead to protracted disseminated infection or be fulminant and rapidly fatal. Septicemic glanders may produce numerous signs consistent with a highly pathogenic bacterial septicemia. Without aggressive treatment, *B mallei* septicemia runs an acute course and may lead to death in 7 to 10 days. The thromboembolic process of glanders was well described by the early 1900s.[1,3] *B mallei* causes damage and subsequent death of the endothelial cells lining the vessels. As the cells detach, the endothelial lining is predisposed to thrombosis. Thrombi serve as an excellent culture medium and seed the bloodstream with bacteria. The embolic process may be realized by the patient as sharp stinging pain in the receiving part or tissue of the body. Robins describes one protracted chronic infection in which the patient was always aware of pain before multiple impending dissemination sites.[3] Bacteremia is transient; however, the more acute or sudden the onset of a septicemic course, the more likely *B mallei* may be isolated from the blood. Bacteremia is also more likely shortly before and during the appearance of multiple eruptions and pustules, if they occur.

Century-old accounts of acute septicemic glanders suggest that virulent organisms and toxins may be so rapidly absorbed that systemic disease is actually primary, preceding the more patent ulcerative and lymphoglandular manifestations. Death may occur before these develop, however. Clinical signs and symptoms of the septicemic process may develop immediately or up to 2 weeks after initial infection or resurgence. These signs and symptoms include any severe constitutional sign and any of the cutaneous, mucous membrane, nervous, and respiratory signs previously discussed. Multiple organs may be involved. Erythroderma, jaundice, severe gastrointestinal distress, abdominal spasm, and severe respiratory signs may develop. Tachycardia, blurred vision, photophobia, excessive lacrimation, altered mental status, hepatomegaly, splenomegaly, granulomatous or necrotizing lesions, and lymphadenopathy may also be present. Death usually occurs in 7 to 30 days without adequate treatment. The prognosis for acute *B mallei* septicemia is guarded regardless of treatment.

Dissemination can also occur in a more benign process resulting in a chronic course, which may be interrupted with latent periods of up to 10 years.[6] Dissemination typically occurs without adequate treatment 1 to 4 weeks after *B mallei* infection of the lymph nodes. The organs most often involved in disseminated infection are the spleen, liver, and lungs, although any can be affected. Other sites include the skeleton, brain, meninges, musculature, and any cutaneous or mucous membrane locations. The kidneys are rarely affected, however. Clinical signs may be absent, limited simply to weight loss, or be highly severe and variable and include any of the aforementioned. Cutaneous eruptions may appear anywhere on the body and often originate from deep pockets of infection in the musculature. The extremities are often affected. Generalized lymphadenopathy with induration, enlargement, and nodularity of regional lymphatic vessels may be found on the extremities and in other affected areas. Miliary abscesses of organs and tissues may resemble tuberculosis. Robins described several cases of disseminated chronic infections in which no clinical symptoms were apparent, yet at autopsy patients were riddled with abscesses, including in the lungs.[3] Robins chronicles a patient with the longest known infection (15 years, only 5 of which were latent) who finally died of disseminated disease. Symptoms of this particular disseminated infection included nasal and aural discharge, submaxillary adenitis, phlegmon of the nose, perforation of the nasal septum, jaundice, diarrhea, and amyloid disease.[3]

The amount of infection and pathology in a surviving patient can be particularly alarming when compared to a usually more rapidly fulminant disease such as septicemic anthrax. Protracted disseminated infections are associated with septic shock and a guarded prognosis. Diagnostic imaging studies are indicated to identify potential infection. Before antibiotics, disseminated infection was ultimately fatal either by resurgence of acute disease or from exhaustion of the patient. Based on the few cases treated with antibiotics,

TABLE 8-2

CLINICAL FEATURES OF EIGHT US LABORATORY-ACQUIRED *BURKHOLDERIA MALLEI* INFECTIONS

Signs and Symptoms*	Patient 1† November 1944	Patient 2† November 1944	Patient 3† February 1945	Patient 4† April 1945	Patient 5† August 1945	Patient 6† August 1945	Patient 7† July 1953	Patient 8† March 2000
Fever, PM rise‡	99.0–99.4	99.0–101.2	101.0–103.4	99.0–103.8	99.0–102.8	-	99.0–101.4	99–104.5
Rigors, chills			+	+				+
Night sweats				+			+	+
Pain in chest	+				+	+	+	
Myalgia	+	+						
Malaise	+		+	+	+	+	+	+
Headache		+	+	+	+	+		
Backache			+	+	+			
Stiff or sore neck			+					
Dehydration	+		+					
Earache			+					
Cough		+			-		+	
Mucopurulent sputum		+						
Oropharyngeal	Postnasal drip	Blister under tongue; nasal obstruction				Sore throat		
Pharynx injected	+	+			+			
Lymphadenopathy	Cervical		Cervical	-	Cervical			L axilla
Neurologic signs			Stupor	Carpo-pedal spasm				
Drowsy			+	+				
Apprehension			+				+	
Dizziness				+				
Fatigue	+	+	+		+		+	+
Weight loss	+						+	+
Anorexia				+			+	
Blurred vision				+				
Lacrimation				+				
Photophobia			+	+				
Abdominal signs			-	Pain L-upper quadrant; spasm		Diarrhea	Indigestion, flatulence, belching	Epigastric tenderness
Nausea, vomiting				+				
Enlarged spleen				+				+
Chest radiographs	R-upper; ~Abscess	R-lower; ~Abscess	R-upper, ~Abscess	Clear	L-middle, ~Abscess	L-lower, pneumo-nitis	L-hilum ~Abscess	Clear

(**Table 8-2** *continues*)

Table 8-2 *continued*

WBC	Normal-low; neutropenia	Normal	High; neutrophilia	High to normal to low; Neutrophils	Normal	Normal to high-normal; Neutrophils	Normal, L-shift; atyp mono, lymph	Normal late in disease
Primary site	Pulmonary	Pulmonary	Pulmonary	Unknown	Pulmonary	Pulmonary	Pulmonary	Cutaneous
Disseminated			Possible	Likely spleen	Possible			+
Secondary sites				Unknown				Liver, spleen
Likely route of entry	Inhalation	Inhalation	Inhalation	Inhalation	Inhalation	Inhalation	Inhalation	Percutaneous
Sputum/throat culture	-		-		-		+	NA
Blood culture	-	-	-	-	-	-	-	+ at 2 mos
Isolation of organism	-	-	-	-	-	-	+	+
CFT positive[§]	Day 50	Day 50	Day 12	Day 40	-	-	-	NA
Agglutinin positive[¥]	Day 50	Day 50	Day 5	Day 23	Day 22	Day 23	Day 19	NA
Mallein test positive	Day 58	Day 58	Day 21	Day 18	Day 72	-	-	NA
Successful treatment	Sulfadiazine 10 days	Sulfadiazine 10 days	Sulfadiazine 36 days	Sulfadiazine 20 days	Sulfadiazine 20 days	Sulfadiazine 20 days	Aureomycin 28 days	Doxycycline 6.5 mos
Onset of antibiotic[¶]	Day 60	Day 60	Days 2, 15, 115	Day 18	Day 16	Day 9	Day 21	~ 5 wks
Recovery time post trx	21 days	Immediate	188 days	12 days	15 days	Immediate	Immediate	> 6.5 mos

*Shaded elements in the table represent the first signs and symptoms according to the medical records of the first seven patients and according to the eighth patient's published case description.
†Patients 1 through 7: Data from original case files. WBC deviations involved only neutrophils. Absolute lymphocyte counts were all normal. Patients 1 and 2: Glanders as a differential diagnosis was delayed. CFTs positive > 10 months, agglutinin titers positive > 10 months, mallein positive > 16 months.
Patient 3: First sulfadiazine treatment was halted because of falling sedimentation rate; two more treatments followed at onset days indicated.
Patient 4: See "Patients 1 through 7" note above.
Patient 5: Eleven normal complete blood counts except occasional slight relative lymphocytosis; lymphadenopathy also at axillary, epitrochlear, and inguinal.
Patient 6: Patient did not take temperature but felt feverish. Agglutinin test considered positive due to titers rising from zero to 1:320.
Patient 7: Previously unpublished case. Early WBC cytology showed transient atypical monocytes and lymphocytes (atyp mono, lymph).
Patient 8: Initial blood culture was negative; data from Srinivasan A, Kraus CN, DeShazer D, et al. Glanders in a military research microbiologist. *N Engl J Med.* 2001;345:256–258.
‡Temperature ranges represent the span of recordings that exceeded normal.
§CFTs were considered positive if >/= 1:20.
¥Agglutinin titers were positive if >/= 1:640 because of background titers in healthy patients of up to 1:320.
¶Onset of antibiotic refers to the day of disease that the successful antibiotics were started; Patient 8 received two prior unsuccessful courses.
+: positive or present
–: negative or not present
[blank]: not reported or no mention
CFT: complement fixation test
mos: months
NA: not applicable or not done
post trx: posttreatment
WBC: white blood cell
wks: weeks

survival is likely if early and long-term effective therapy is instituted. Even with treatment, clinical symptoms may continue several months before complete resolution, particularly if treatment is delayed.

Radiographic imaging is useful to monitor pulmonary infection. Early radiographic signs are typically infiltrative or support early abscess formation. Segmental or lobar infiltrates are common. With time, pulmonary abscesses tend to undergo central degeneration and necrosis, which radiographically resembles cavitation, and these may be single or multiple. Unilateral or bilateral bronchopneumonia may be seen, as well as a smattering of miliary nodules. Because of the potential for disseminated disease,

computed tomography scan is useful for monitoring deep tissues and visceral organs.

Complete blood count and chemistry studies for glanders patients vary depending on the disease's location of infection and duration, and the degree of dissemination or septicemia. Complete blood count may be normal early and throughout the pretreatment disease course. Based on the laboratory-acquired cases, deviations in the white blood cell count typically involve only the absolute neutrophil count rather than other cell lines (Table 8-2). Neutropenia or neutrophilia, with or without a left shift, may be transient findings. Leukcopenia with mild to moderate relative lymphocytosis was seen in three of the six laboratory-acquired infections reported by Howe and Miller,[5] which may be attributed to a low absolute neutrophil count. Absolute lymphocyte counts were consistently within normal limits.

Historically, mortality rates have been reported to be 95% without treatment and up to 50% with treatment. A more recent analysis estimates the mortality rate for localized disease is 20% when treated, and the overall mortality rate is 40%.[65] Since the near eradication of glanders and the development of effective antibiotics, even these may be high estimates. Successful cure was achieved in 100% of the eight US laboratory-acquired cases, despite three of the eight cases (37%) experiencing a delay in effective treatment of 2 months.[5,11,56] A brief period of "apparent recovery" is a common clinical feature that can easily lead to delayed treatment and complications. Four of the eight patients were successfully treated with sulfadiazine for at least 20 days. The first two who received delayed treatment still recovered with only 10 days of sulfadiazine, yet recovery was protracted. The most recent patient (patient 8) had disseminated disease, which included abscesses of the spleen and liver, and required ventilatory assistance before improving on a prolonged course of several antibiotics. These recent cases imply that prognoses range from good with localized infection and prompt treatment to guarded with septicemic infection.

Laboratory Diagnosis

Morphology and Growth Characteristics

A definitive glanders diagnosis in humans occurs when the organism is isolated in culture and correctly identified. In endemic regions, phenotypic characteristics such as colony and cell morphology in combination with biochemical assays may still be a practical means to definitively diagnose glanders. These methods may take 2 to 7 days to confirm a diagnosis.[139] Gram stains alone of pus from lesions may be productive, but microorganisms are generally difficult to find or isolate. *B mallei* can be cultured and identified with standard bacteriological media.[46] In potentially contaminated samples, supplements to inhibit the growth of gram-positive organisms (eg, crystal violet, bacitracin, penicillin) and some gram-negatives (eg, polymyxin B) and facilitate selective isolation of *B mallei* can be useful.[140,141] The optimum growth temperature is approximately 37°C. Growth is typically slow on nutrient agar, but is more rapid (2 days) when enhanced with 1% to 5% glucose and/or glycerol, and on most meat infusion nutrient media.[140,142] *B mallei* colonies typically are smooth and about 1 mm in width, white (turning yellow with age), semitranslucent and viscid on Loeffler's serum agar and blood agar. After incubating for 3 days on sterile potato slices, growth appears as a shiny, moist, yellowish transparent film.[24,143] Selective differentiation from the related organisms *B pseudomallei* and *Pseudomonas aeruginosa* may be achieved by examining the following phenotypes. Whereas *B mallei* does not grow at 42°C or at 21°C or in the presence of 2% sodium chloride, *B pseudomallei* and *P aeruginosa* do. Also, it has been reported that *B mallei* does not grow on MacConkey agar, whereas both *B pseudomallei* and *P aeruginosa* grow.[7,24] However, others found that *B mallei* strains grew on this agar as nonlactose fermenting colonies.[24]

B mallei is a small, nonsporulating, aerobic gram-negative bacillus approximately 2 to 4 μm long and 0.5 to 1 μm wide. It is nonmotile, a characteristic that differentiates it from related organisms such as *B pseudomallei*. The presence of a thick polysaccharide capsule can be demonstrated on the surface by immuno-electron microscopy.[77] In the presence of nitrogen, the organism can grow as aerobic and facultative anaerobe.[22,24,144] Size may vary by strain and by environmental factors including temperature, growth medium, and age of culture. Organisms from young cultures and fresh exudate or tissue samples typically stain in a bipolar fashion with Wright stain and methylene blue. Organisms from older cultures may be pleomorphic.[140] In vivo *B mallei* is found most often to be extracellular. Since the disease is rare, samples should be designated as "glanders suspect" until confirmed by more extensive testing. Sample security to include appropriate chain-of-custody documentation is also prudent for all samples.

Isolation

The isolation of *B mallei* in culture is the gold standard for a glanders diagnosis. However, it can be difficult to obtain clinical specimens harboring viable bacteria; and invasive techniques, such as aspiration

and biopsy, may be required. Even then, *B mallei* bacteria are often difficult to find, even in acute abscesses. Although isolation from blood has sometimes been successful in acute human cases, blood cultures are frequently negative until the disease's terminal stages and do not generally appear to be a reliable indicator of infection, at least in animals such as NHP (Patricia L Worsham, David M Waag, and Taylor B Chance, USAMRIID, Fort Detrick, MD, unpublished data, 2013; Samuel L Yingst and Mark J Wolcott, USAMRIID, Fort Detrick, MD, unpublished data, 2013).[24,44,56] To amplify the presence of low numbers of bacteria in normally sterile sites, animal inoculation methods were often used previously. However, such studies are impractical and inadvisable now for several reasons:

- the time required for disease to manifest;
- logistical requirement for special containment facilities;
- stringent current animal regulatory requirements; and
- adverse public reception of such animal work.

Isolation of the agent in nonendemic regions or from potentially contaminated samples may require use of selective media. Several media are commercially available for isolation of human *Burkholderia* pathogens, such as BCA (*Burkholderia cepacia* agar also referred to as PC [*Pseudomonas cepacia*] agar), OFPBL (Oxidative-Fermentative-Polymyxin B-Bacitracin-Lactose agar), and Burkholderia Cepacia Selective Agar. These and other media have been described previously.[145–147] For example, BCA was originally developed as a selective medium to isolate *Burkholderia cepacia* complex from the sputum of individuals with cystic fibrosis. Commercial preparations typically contain crystal violet, bile salts, ticarcillin, and polymyxin B (Remel, Lenexa, KS). It was found to be sensitive and selective for both *B pseudomallei* and *B mallei*.[146,147] OFPBL agar is another commercially available selective medium used to isolate *B cepacia* from cystic fibrosis patients. It permitted growth of 80% of strains tested, even though the colonies are very small and translucent; the growth often causes the agar to change from green to yellow due to lactose fermentation.[146] OFPBL can be used in conjunction with (but not in place of) BCA agar for selective isolation of *B mallei* or *B pseudomallei*. Although both media are significantly discriminating for *B mallei* and *B pseudomallei*, they are not totally selective and strains of *B cepacia*, other *Burkholderia*, and some non-*Burkholderia* organisms can be expected to be isolated with them.[146]

Identification

Biochemical Identification. Automated biochemical kits are available commercially, such as API 20NE (bioMerieux, Durham, NC), RapID NF (Remel, Lenexa, KS), VITEK (bioMerieux, Durham, NC), and Biolog Inc phenotype microarray systems; however, they have often misidentified the *Burkholderia*. In 2012, USAMRIID Supervisory Research Microbiologist Mark Wolcott relayed in several written and oral communications that *B mallei* and *B pseudomallei* have been misidentified as nonpathogenic bacteria or other pathogens such as the *B cepacia* complex or *Pseudomonas*.[24,56,148,149] This situation was exemplified with the most recent case of human glanders in which the infecting *B mallei* strain was identified by an automated bacterial identification system as *Pseudomonas fluorescens* or *Pseudomonas putida*.[56] Another drawback is that many of these methods require the organism to be cultured in vitro before testing, resulting in a delay in the diagnosis.

The MIDI Sherlock Microbial Identification System (Microbial Identification System, version 4; MIDI Inc, Newark, DE) can identify isolates of the *Burkholderia* by gas–liquid chromatography of cellular fatty acids. Inglis obtained good results using MIDI with *B pseudomallei*; and gas–liquid chromatography was used to correctly classify the *B mallei* from the most recent case of human glanders in the *Burkholderia*.[56,150] However, the bacteria must first be cultured under specific standardized conditions, and sample preparation is laborious and time-consuming. It does not usually allow speciation of the *Burkholderia* because of, for example, the highly similar cellular fatty acid compositions.[53] Yet specific fatty acids and derivatives, such as methyl esters, are being identified that appear to be *Burkholderia* species-specific.[148,151]

Nucleic Acid-based Identification

A major obstacle to isolating the pathogenic *Burkholderia* directly from samples is their low concentration in tissues and biological fluids of infected hosts. The development of methods for the reliable detection of glanders that does not rely on isolation of the organism is especially important for the diagnosis of chronic glanders. Therefore, many attempts have been made to develop indirect assays, such as nucleic acid analysis. The genomes of at least nine strains of *B mallei* have been sequenced,[152] and the data are being used to enhance the ability to specifically identify this microorganism and increase understanding of how *B mallei* interacts with its host.[38] Several nucleic acid-based diagnostic methods can confirm specific identification of *B mallei*,

often within several hours. Whereas some of the DNA-based procedures reported could be performed directly on clinical samples, others required preliminary culturing to isolate the bacteria. Many of the methods include polymerase chain reaction (PCR)-based and DNA gene sequencing-based assays.[153–155] The latter have included the 16S and 23S rRNA-encoding genes, S21 ribosomal protein gene loci sequences, and MLST procedures. For instance, Frickmann et al showed that the sequence comparison of a 120 base pair ribosomal protein S21 gene fragment was useful as a diagnostic procedure for the discrimination of *B mallei* and *B pseudomallei* from the nonpathogenic *B thailandensis* and several other environmental species of *Burkholderia*, but it did not differentiate between *B mallei* and *B pseudomallei*.[156] Gene fragments and a single nucleotide polymorphism in the 16S rRNA gene have been reported to differentiate *B mallei* from *B pseudomallei*.[155,157] Analysis of the 16S ribosomal RNA gene sequence analysis identified *B mallei* from other *Burkholderia* species in the 2000 US laboratory-acquired infection.[56] However, it will be necessary to analyze many different species of *B mallei* and *B pseudomallei* (and other species) to establish the specificity of these and similar single-locus typing procedures. For discrimination of the closely related species *B mallei* and *B pseudomallei*, sequencing of the entire 16S rRNA gene, sequencing of the 16S-23S rRNA intergenic spacer, or the addition of a specific PCR or other genotyping method to a 16S rRNA gene fragment test has been recommended.[158]

PCR-based techniques and DNA gene sequencing are increasingly used in clinical settings and public health laboratories for bacterial identification.[159] Automation of sequencing and improved reagents have also reduced the cost per test and the time required for identification. Furthermore, because killed bacteria or their templates may be used, these techniques also have the advantage of reducing the risk of exposure and infection to laboratory personnel compared to conventional methods.[153] The current interest in biowarfare defense research prompted an increased capability based on recent publications.[153–155,157,160–165] Numerous PCR assays have been described, some of which were recently described in detail in a review on PCR methods.[166] They target various genetic elements, such as specific insertion sequences or single nucleotide polymorphisms, secretion system genes, and flagellar biosynthetic genes.[157,162–165] Most of these assays require evaluation using many more diverse strains of the pathogenic *Burkholderia* and related/unrelated species and testing for diagnostic applicability in a controlled infection study, such as an animal natural history study, for full validation. For instance, a real-time PCR assay, BurkDiff, was designed to target a unique conserved region in the *B mallei* and *B pseudomallei* genomes containing a single nucleotide polymorphism that differentiates the two species.[157] Assay sensitivity and specificity were assessed and confirmed by screening BurkDiff across 469 isolates of *B pseudomallei*, 49 isolates of *B mallei*, and 390 nontarget isolates. The agreement of results with traditional identification methods and lack of cross-reactivity prompted the suggestion that BurkDiff may be a robust and specific assay for the detection and differentiation of *B mallei* and *B pseudomallei*; however, test results may be difficult to interpret and the assay must be assessed for its diagnostic applicability in controlled models for *B mallei* infection.[167]

Several PCR assays have targeted genes of the T3SS or other secretion systems; these multigenic systems have been shown to be important in the pathogenesis of *B mallei* and *B pseudomallei*.[165,166,168–171] Two assays based on the bimA (*Burkholderia* intracellular motility A) gene of the type V secretion system were developed.[162,163] It was demonstrated that the N-terminal nucleotide sequence of bimA contains a unique *B mallei* region not present in the *B pseudomallei* and *B thailandensis* bimA genes, as verified in tests with numerous strains of these three species.[162,163] The value of the assays for early, rapid, and specific diagnosis of glanders in mice infected by the aerosol route was shown.[162] However, it was reported later that the bimA of *B pseudomallei* strains from Australia contain an N-terminus identical to that in *B mallei* bimA and a *B thailandensis*-like strain was detected in a bimA-based PCR assay.[109,166,172] Finally, other PCR assays based on flagellar biosynthesis proteins (such as fliP) have been shown to be highly sensitive in tests with *B mallei*.[29,166,173,174]

Because of potential problems intrinsic to PCR assays such as false positives, gene mutation, and PCR inhibitors, some have recommended the use of two DNA targets (two PCR assays) in combination with sequencing of the amplicons.[52] Several multiplex PCR assays that target several and partially alleviate these issues have been developed. For example, Lee, Wang, and Yap described a sensitive and specific multiplex PCR using a short variable copy repetitive sequence, a metalloprotease gene fragment, and a sequence unique to *B thailandensis* that could distinguish *B mallei*, *B pseudomallei*, and *B thailandensis*.[175] Koh et al separated the species *B mallei*, *B pseudomallei*, *B thailandensis*, and the *Burkholderia cepacia* complex by using four specific primers: (1) a putative sugar binding protein, (2) a hypothetical protein, (3) a putative outer membrane protein, and (4) 16S rDNA.[176]

Other DNA-based techniques using pulsed-field gel electrophoresis and ribotyping have been used to identify strains of *B pseudomallei* and differentiate

their virulence; these methods have not been tested with *B mallei* and would likely be more time and labor intensive than gene sequencing.[177,178] More recently, improved in silico probe design based on short unique regions of the target genome has aided in the potential use of microarray procedures to selectively distinguish *B mallei* and *B pseudomallei* genetically; more microarray tests of these genetic targets with many bacterial strains are needed.[179]

Immunological Detection

Immunoassays that detect the presence of specific microorganisms can be useful in disease diagnosis. No such tests specifically for *B mallei* are established despite advancements in immunodetection of *B pseudomallei*.[180,181] Similar efforts to develop *B mallei*-specific immunodetection methods are important because antibiotics that are efficacious for one disease might not be effective for the other. mAbs elicited by *B mallei* whole cells and that targeted the LPS have been described.[104] They appeared to be specific for *B mallei*, failing to recognize *B pseudomallei,* and might be useful reagents for a direct immunoassay for *B mallei*. In related studies, two groups developed large panels of mAbs to capsule polysaccharide and LPS that were specific for *B mallei*, *B pseudomallei,* or both and demonstrated strong binding to the bacteria.[99,165] The study by Zou et al also revealed additional mAbs of possible diagnostic value, specifically, a pathogenicity-linked antigen epitope(s) on capsule-like polysaccharides found only in the pathogenic species of *Burkholderia* (both *B mallei* and *B pseudomallei*), and several *B mallei* LPS-specific mAbs.[99] It is possible that by using a combination of mAbs from different antigen groups, different strains of *B mallei* and *B pseudomallei* can be effectively differentiated from each other and from other nonpathogenic *Burkholderia* species.

Serologic Diagnosis

Although serological tests have been developed for diagnostic use in equines, no such tests exist to identify glanders specifically in humans. The mallein skin test has been primarily used to detect glanders in horses.[24,182] A human version of the skin test was of little diagnostic value because of the multiweek delay to obtain a positive result. However, modified tests yielded somewhat improved results. In eight laboratory-acquired, confirmed cases of human glanders in the United States, the test was negative in two, not completed in one, and first positive in five on days 18 to 72 postinfection.[177] Overall, it appears that this diagnostic test for human glanders is minimally useful.

In vitro tests to include the indirect hemagglutination assay (IHA) and complement fixation test (CFT) have been used for serologic glanders detection. The IHA, which is the most frequently used serological test for human melioidosis, can also be used to identify glanders cases.[183–185] In melioidosis testing, the failure of the IHA to detect antibody responses despite culture-confirmed disease has been observed.[183] The CFT is still used universally in veterinary medicine as a reasonably reliable and low-cost procedure for animal glanders diagnosis.[44,186–188] However, the CFT can be nonspecific and may not detect all cases or stages of glanders. In addition to occasional false negative results, it has also produced frequent false positive results (low specificity) and been hampered by inhibitory effects on complement of sera.[24,183,186,189] The major problem leading to the low sensitivity and specificity of the CFT and other in vitro procedures has been linked to the test antigens currently used, that is, crude preparations of whole cells.[44,190] Use of such antigens has led to frequent false positive results resulting from cross-reactive antigens. To address these shortcomings, numerous new tests have been reported for animal diagnosis (described below); however, improved assays are also clearly needed for human glanders serodiagnosis.

Several assays and reagents have been described recently for the improved serodiagnosis of human glanders. Waag et al developed a whole cell enzyme-linked immunosorbent assay (ELISA) using irradiation-killed *B mallei*.[44,191] The test identified patients that have melioidosis or glanders (and excluded other differential diagnosis candidates, ie, anthrax, brucellosis, tularemia, Q fever). Similarly, Parthasarathy et al developed a polysaccharide microarray using extracted CPS and LPS to facilitate specific detection of *B pseudomallei* and *B mallei* antibodies in animal and human sera.[192] However, neither assay can discriminate between the two *Burkholderia* diseases because of serological cross-reactivities.[56,191,192] By using bioinformatic or similar state-of-the art approaches, others have identified *B mallei*-specific proteins that have potentially improved prospects for glanders-specific serodiagnosis.[190,193] For example, Varga et al used pre-and postexposure sera from 2,000 cases of human glanders and a protein array platform made for studying melioidosis patients to characterize the human immunological response to *B mallei*.[56,191,193–195] Significantly increased antibody responses to 17 of 156 peptides were detected and antibodies to only two (a pilus biosynthetic protein and 50S ribosomal protein) were shared between the two diseases, implying that the human antibody response to *B mallei* is markedly distinct from that to *B pseudomallei*. The results of these recent studies

suggest that these antigens may be useful for an improved glanders diagnosis; however, additional studies to include nonspecific and glanders-specific sera from human (and animal model) sources are needed.

Other Identification Methods

New methods to verify identification of isolated organisms as exemplified by phage-based identification have been described. Bacteriophages have been isolated which exhibit infectivity and high specificity for *B mallei*. For instance, φE125 and φ1026b infect *B mallei*, but not *B pseudomallei*.[196,197] Efforts to develop phage variants with species-specific receptors offer the potential for convenient phage-based diagnostics. Mass spectrometry methods, such as matrix-assisted laser desorption/ionization mass spectrometry and Raman spectroscopy, are being exploited for the identification of *B mallei* and *B pseudomallei*.[198,199] For example, signatures specific to pathogens at the species level were developed to evaluate a Raman chemical imaging spectroscopy method for reagentless detection and identification.[198] Raman chemical imaging spectroscopy combines Raman and fluorescence spectroscopy and digital imaging to allow detection of low levels of biothreat organisms in the presence of complex environmental backgrounds without prior amplification methods. Raman spectra for viable select agents and toxins including *B mallei* and *B pseudomallei* were reported; however, most of the studies were conducted to distinguish *Burkholderia* from other pathogens (eg, *B anthracis*) and more efforts to distinguish near-neighbors of *B mallei* are needed. Other new assays for direct detection of the organism in culture and tissues and that use probes for surface or secreted antigens of the pathogenic *Burkholderia* are also being evaluated.[133,200] One example is a fluorescence in situ hybridization assay that correctly identified all of the *B mallei*, *B pseudomallei*, and *B thailandensis* strains.[133] However, the assay requires a relatively high bacterial concentration; thus, fluorescence in situ hybridization analysis may be more useful for evaluating tissue sections in pathogenesis studies than for diagnosis of direct clinical samples.

New combination methods are being developed. An assay coupling biothreat group-specific PCR with electrospray ionization mass spectrometry and using DNA extracts from killed bacteria correctly identified seven bacterial biothreat bacterial species (including *B mallei* and *B pseudomallei*) to the genus if not species level.[201] A gas chromatography-mass spectrometry method was used to identify several cellular fatty acid methyl ester fragments that could differentiate the species *B mallei*, *B pseudomallei*, and *B thailandensis*.[148]

Diagnosis in Equids

Several diagnostic tests have been developed and used extensively for glanders diagnosis in equines.[24,53] Khan et al provides a comprehensive review and evaluation of diagnostic tests developed previously as well as an overview of recent assays aimed at improving the sensitivity and specificity of glanders diagnosis.[24] Tests used currently consist primarily of serodiagnostic assays, that is, the CFT and the mallein skin test. Serum anti-*B mallei* antibodies are detected by the CFT within a week of infection, and the CFT can detect carriers and animals chronically infected with *B mallei*[24,189]; it is the only mandatory serological test for international trade of equids, but it can be hampered by the problems detailed in the previous section.[22,187] The mallein test is another well-known and established test used for glanders diagnosis in animals and involves injecting a purified protein derivative of the *Burkholderia* glycoprotein mallein intradermally. An immune cellular response as manifested by a delayed type hypersensitivity reaction is observed and considered diagnostic for glanders.[24,177,182]

More recently developed assays include competitive and indirect ELISAs using *B mallei*-specific monoclonal Abs, ELISAs with purified recombinant proteins used as test antigens, and Western blot techniques.[189,202,203] Using sera from horses in an endemic region of South East Asia, Elschner and Khan et al reported the significantly improved specificity and sensitivity of the CFT when it was combined with a complement independent immunoblot technique, an approach that increased both the detection rate and specificity of the test for glanders serodiagnosis.[189,202] Kumar et al cloned a novel recombinant *Burkholderia* intracellular motility A (rBimA) protein and used it with many positive and potentially negative serum samples in an indirect ELISA to detect equine glanders.[203] The results revealed 100% sensitivity and 98.9% specificity. Also, rBimA protein did not react with melioidosis patient and normal healthy human serum samples, and thus showed high specificity.

Treatment

Because human cases of glanders are rare, limited information exists regarding the use of modern antibiotic treatment for humans. *B mallei* infection responds to antibiotic therapy; however, recovery may be slow after a delayed diagnosis or with disseminated disease. Reports in the scientific literature indicate that most strains of *B mallei* are susceptible to the following antibiotics in vitro:

- amikacin,
- netilmicin,
- gentamicin,
- streptomycin,
- tobramycin,
- azithromycin,
- novobiocin,
- piperacillin,
- imipenem,
- ceftazidime,
- tetracycline,
- oxytetracycline,
- minocycline,
- doxycycline,
- ciprofloxacin,
- norfloxacin,
- ofloxacin,
- enrofloxacin,
- erythromycin,
- sulfadiazine,
- trimethoprim/sulfadiazine,
- trimethoprim/sulfmethoxazole (co-trimoxazole), and
- amoxicillin-clavulanate (co-amoxiclav).[89,204–212]

Aminoglycosides and other antibiotics incapable of penetrating host cells probably will not be useful in vivo because *B mallei* is a facultative intracellular pathogen.[204,205,211] Susceptibility to streptomycin and chloramphenicol in vitro has been inconsistent, with some reporting sensitivity and others reporting resistance.[7,205,209,212] *B mallei* is susceptible to the lytic action of human granulysin, a broad-spectrum antimicrobial peptide, and silver containing compounds.[213,214]

Most *B mallei* strains exhibit resistance to the following antibiotics:

- amoxicillin,
- ampicillin,
- penicillin G,
- bacitracin,
- chloromycetin,
- carbenicillin,
- oxacillin,
- cephalothin,
- cephalexin,
- cefotetan,
- cefuroxime,
- cefazolin,
- ceftriaxone,
- metronidazole, and
- polymyxin B.[7,11,47]

In addition, a study focused on 41 isolates of *B mallei* obtained from various outbreaks of equine glanders occurring between 1999 and 2006 in Punjab, Pakistan, reported that less than 50% of the isolates were resistant to oxytetracycline, roxithromycin, and norfloxacin, and less than 40% were resistant to ciprofloxacin.[212] Consistent with the published literature, all of the Pakistani isolates were susceptible to co-amoxiclav, chloramphenicol, doxycycline, gentamicin, and trimethoprim-sulfamethoxazole, and approximately 95% were susceptible to both enrofloxacin and ofloxacin.

Antibiotics have been tested against glanders in hamsters, mice, equids, guinea pigs, and monkeys.[207,208,211,215–217] Sodium sulfadiazine—but not penicillin or streptomycin—was effective for treating acute glanders in hamsters.[208] Doxycycline and ciprofloxacin were also examined in the hamster model of glanders.[211] Doxycycline therapy was superior to ciprofloxacin therapy, but relapse did occur in some of the treated animals 4 to 5 weeks after challenge. Hamsters were also infected subcutaneously or by aerosol with *B mallei* and were treated with ofloxacin, biseptol, doxycycline, and minocycline.[215] All of the antibiotics exhibited some activity in animals challenged subcutaneously, but ofloxacin was superior. None of the antimicrobials demonstrated appreciable activity against a high dose of *B mallei* delivered by aerosol, but doxycycline provided 70% protection against a low dose delivered by this route.[214]

Ceftazidime and levofloxacin were examined as treatments for glanders in BALB/c mice infected intranasally with *B mallei* ATCC 23344.[218] Despite good in vitro activity against *B mallei*, intraperitoneal delivery of the antibiotics failed to eradicate the organism and resulted in the development of nonlethal, chronic glanders. More recent studies using a BALB/c model of inhalational glanders reported that oral administration of co-trimoxazole twice daily for 14 days prevented the development of acute disease, but was not able to completely eliminate *B mallei* and ultimately resulted in the establishment of a chronic infection.[89] Co-trimoxazole is recommended for postexposure prophylaxis in humans; however, an extended course of therapy of up to 21 days is likely to be indicated.[89,219,220]

In most countries, strict regulations mandate that animals testing positive for glanders are destroyed rather than treated. Given the difficulties in implementation of such regulations in some countries along with the high monetary value of horses in equestrian sport and breeding, the usefulness of modern antibiotics for treatment or postexposure prophylaxis of horses has recently been reevaluated. In a 2012 report Saqib et al described the effectiveness of antibacterial therapy on 23 culture positive horses involved in a

confined glanders outbreak at the Lahore Polo Club in Pakistan.[37] A treatment protocol was implemented consisting of once daily parenteral administration of enrofloxacin and trimethoprim/sulfadiazine followed by twice daily oral administration of doxycycline for 12 weeks (84 days). All horses showed a marked improvement during the initial week of treatment. Abatement of fever, renewal of appetite, and healing of ulcerated nasal septa were reported by the end of week 1. Nodules and ulcers were cleared in most horses by the end of week 3. No clinical signs were observed in any horse following week 12. To confirm the absence of disease, immunosuppression was induced (by daily corticosteroid injection for 10 days) in six randomly selected horses starting on day 90 following completion of therapy. No recrudescence of disease as determined by a lack of clinical signs was observed. By 8 months posttreatment, the IHA titers of all of the horses were considered to be negative (≤1:320). At the time of the report (September 2012), nine of the horses in the study were still at the Lahore Polo Club and remained healthy; the status of the remaining eight was unknown. Whereas the findings of the Saqib et al are promising, the widespread treatment of glanderous horses is unlikely to replace the practice of "testing and culling" because the latter is a more cost-effective and efficient means of containing the disease spread. This study does, however, provide important information that may be applicable in certain situations where treatment is desirable.

The majority of human glanders cases occurred before the antibiotic era, and more than 90% of cases resulted in death.[221] Several human glanders cases have been recorded since the 1940s, primarily in laboratory workers, and these have been successfully treated with antibiotics.[5,56,222,223] Sulfadiazine was used successfully in the first six US laboratory-acquired infections.[5] The seventh was successfully treated with the tetracycline compound, aureomycin. Two additional cases were successfully treated with sulfadiazine in 1949 and 1950.[222] Disseminated glanders in a stablehand who had only indirect contact with horses was also treated successfully with aureomycin in Austria in 1951.[55] Streptomycin was used to treat a patient infected with *B mallei* and *Mycobacterium tuberculosis*.[223] Treatment with streptomycin reportedly cured the glanders, but had little effect on the bone's tuberculosis in this patient. In a recent case of laboratory-acquired glanders, the patient received imipenem and doxycycline intravenously for 1 month followed by oral azithromycin and doxycycline for 6 months.[56] Susceptibility testing of the *B mallei* isolate in this case demonstrated sensitivity to the former two drugs, although retrospective susceptibility testing found that the organism was resistant to azithromycin.[51] Diagnostic imaging of the patient's splenic and hepatic abscesses through the 6-month course showed their near complete resolution.

No Food and Drug Administration-approved therapy for glanders exists. Recommendations for antibiotic therapy depend on the infection site and severity. Localized disease should be treated with at least a 2-month—and preferably a 6-month—course of antibiotics based on sensitivity. Without susceptibility test results and for mild disease, oral doxycycline (100 mg twice/day) plus trimethoprim-sulfamethoxazole (4 mg/kg/day in two divided doses) for 20 weeks is recommended.[224] Historically, oral chloramphenicol has been added to this regimen for the first 8 weeks; however, based on recent recommendations for melioidosis treatments, this may no longer be necessary. Amoxicillin/clavulanate is the recommended alternative for pregnant women and children or individuals that cannot tolerate trimethoprim-sulfamethoxazole. For severe disease ceftazidime at 40 mg/kg intravenously (IV) every 8 hours or imipenem IV at 15 mg/kg every 6 hours (maximum 4 g/day) or meropenem at 25 mg/kg IV every 8 hours (maximum 6 g/day) plus trimethoprim-sulfamethoxazole at 8 mg trimethoprim/kg per day IV in four divided doses is recommended. Intravenous therapy should be continued for at least 14 days and until the patient is clinically improved. Oral maintenance therapy as for mild disease can be continued from that point.[224] Combined therapy for at least the first month should be considered for patients even with the mildest of systemic symptoms. For visceral and severe disease, prolonged treatment up to a year is recommended. Abscesses may be surgically drained, depending on their location.[65] Because of the intractable nature of glanders, long-term follow-up and possibly prolonged, tailored therapy is indicated for infections that are slow to clear. Patients should be followed at regular intervals for at least 5 years after recovery. Diagnostic imaging is useful to follow the reduction and resurgence of abscesses, serology may help to monitor the clearing of antibody, and inflammatory markers may also suggest resurgence of a latent infection. Patients should be advised of the lifelong risk of relapse and to alert their healthcare providers of their previous history, particularly if they develop a febrile illness. This situation becomes even more important when potentially dealing with a genetically engineered strain of *B mallei*. Current postexposure prophylaxis recommendations for laboratory workers consist of oral trimethoprim-sulfamethoxazole for 21 days.[219,220,224] If the patient is allergic to or intolerant of this, or the organism is known to be resistant to the first choice, the second-line choice is oral amoxicillin/clavulanic acid (co-amoxiclav) for 21 days.[219]

Prophylaxis

Host Immunity

B mallei can establish chronic or acute infections in multiple species, which suggests that native host immune responses are unable to eradicate wild type organisms upon initial challenge. In addition, no evidence indicates previous infection provides immunity against glanders.[7,225] Infections in horses that appeared to symptomatically recover from glanders would recrudesce when the animals were challenged with *B mallei*. Inoculation of *B mallei* into chronically infected horses produced at least local infections most of the time, and occasionally a manifestation of classic glanders. Similar to equines, protective immunity in humans after recovery from glanders is not believed to occur. In an 1869 human case report from Poland as told by Loeffler, one attempt at autoinoculation with the fluid from a pustule produced more pustules.[1] Thus, patients who recover may still be susceptible, making reuse of the agent in biowarfare necessary to consider.

Immune responses to glanders appear to be complex requiring both humoral and cell-mediated immune (CMI) responses. The role of antibodies in immune protection has been investigated experimentally in animals as well as retrospectively in a recent human case. Experiments on horses regarding protective immunity have given ambiguous results.[1,7] Passive immunity experimentation using equine sera has also failed.[7] Conversely, passive immunization of BALB/c mice using *B mallei* OPS- or CPS-specific mAbs conferred nonsterilizing protection against a lethal bacterial challenge delivered either intranasal or by aerosol.[97,104] Consistent with a role for antibodies in protection against glanders, investigations by Whitlock et al involving the depletion of B cells in BALB/c mice demonstrated a significantly decreased survival time following *B mallei* infection compared to control mice.[226] In contrast, Rowland et al reported that when B cell knockout mice (μMT C57Bl/6 mice) were infected with *B mallei*, survival of B cell deficient animals did not differ from wild type mice.[227] The reason for these contrasting results is unclear, but it is possible that these differences may be a consequence of the manner in which the B cells were depleted (ie, antibody depletion versus genetic deletion) or the specific mouse models used.

Analysis of humoral immune responses from a laboratory-acquired case of glanders in 2000 indicated that *B mallei* specific immunoglobulin A, immunoglobulin G, and immunoglobulin M levels were highly elevated (8-, 16- and 4-fold, respectively) by 2 to 4 months postinfection and then began to decline.[191] By 14 months postinfection antibody titers returned to near baseline levels. More extensive characterization of the antibody responses in this case demonstrated reactivity of highly increased (≥2-fold compared to preexposure serum) antibodies with a variety of *B mallei* and/or *B pseudomallei* proteins including T3SS and T6SS components, type IV pili, outer membrane proteins, chaperones (eg, GroEL and GroES), and hypothetical proteins.[193] Screening of equine glanders serum against a bacteriophage expression library revealed the presence of antibodies to a *Burkholderia* Hep_Hag autotransporter (BuHA) proteins, a family of immunodominant antigens predicted to be hemagglutinins and invasins.[123] Such proteins represent potential candidate antigens to develop glanders diagnostics, therapeutics, and vaccines.

Since *B mallei* is a facultative intracellular pathogen, CMI mechanisms as well as cytokine and chemokine expression, significantly contribute to the clearance of the organism from infected hosts. *B mallei* is capable of infecting and surviving in many cell types including professional phagocytes. In a BALB/c model of glanders, neutrophils and macrophages infiltrated the spleen 5 hours postinfection and an increase in activated macrophages, neutrophils, and T cells occurred by 24 hours postinfection.[227] When neutrophils were depleted, mice became acutely susceptible to *B mallei* infection and succumbed within 5 days. In contrast, if mice were depleted of both $CD4^+$ and $CD8^+$ T cells, they did not succumb until 14 days postinfection. In wild type C57Bl/6 mice, macrophages have been shown to be important for reducing the susceptibility of the animals to pneumonic *B mallei* infection.[82,228] Monocyte chemoattractant protein-1 (MCP-1), a chemokine involved in the chemoattraction of macrophages to sites of infection, plays an important role in protective immunity to *B mallei* infection. Mice lacking either MCP-1 or the MCP-1 receptor were more susceptible to disease following IN challenge and exhibited higher bacterial burdens in organs at 3 days postinfection compared to wild type mice.[82] Monocyte and inflammatory dendritic cell recruitment was defective in the MCP-1 knockout mice and increased numbers of neutrophils were observed in the lungs. These data support a critical role for phagocytic cells (neutrophils, monocytes and macrophages, and dendritic cells) in controlling the early, innate responses to *B mallei* infection while T cells appear to be important later in infection.[227]

Interferon-gamma (IFN-γ) plays an important role in macrophage activation and clearance of intracellular organisms. Examination of cytokine responses 24 hours following IP injection of female BALB/c mice with *B mallei* exhibited a strong IFN-γ response and

elevated levels of IL-18, IL-12, IL-27, IL-6, and MCP-1.[229] IFN-γ knockout mice infected with *B mallei* died within 2 to 3 days after infection, and uncontrolled bacterial replication in several organs confirmed a critical role for this cytokine during innate immune responses to *B mallei*. Similar findings were reported when specific antibodies were used to deplete IFN-γ in mice.[226] The proinflammatory cytokines IL-12 and IL-18 were shown to be critical for IFN-γ production at early time points postinfection.[229] Neutralization of IL-12 in vivo led to increased susceptibility of mice to lethal infection, possibly from its role in promoting IFN-γ production by natural killer cells and T cells.[230] Consistent with this notion, in vitro assays have confirmed that natural killer cells and $CD8^+$ T cells were the main cellular sources of IFN-γ generated in response to *B mallei*.[229] Furthermore, MyD88 knockout mice (which cannot produce IFN-γ) were also highly susceptible to pulmonary challenge with *B mallei* compared to wild type mice.[228] Treatment of MyD88 knockout mice with exogenous recombinant IFN-γ helped to restore effective immunity and significantly increased survival.[82,228] Thus, *B mallei* appears to be susceptible to CMI responses promoting the expression of type 1 cytokines (eg, IFN-γ and IL-12). Although the initial burst of IFN-γ was able to control bacterial replication, clearance was not achieved.[229] These studies highlight the importance of cytokine and chemokine production during *B mallei* infection and suggest that strategies targeting the production of IFN-γ should be considered when attempting to achieve effective vaccine induced immunity.

LPS and various microbial products can activate macrophages through TLRs and other pattern-recognition receptors. Pattern-recognition receptors signaling leads to transcription of genes encoding cytokines, chemokines, and enzymes, such as inducible nitric oxide synthase that aids in the clearance of intracellular bacteria. Purified *B mallei* LPS is a strong activator of human TLR4 complexes and stimulates production of tumor necrosis factor-alpha (TNF-α), IL-6, and regulated upon activation normal T-cell expressed, and secreted (RANTES) protein in human macrophages and dendritic cells.[98] The expression of these cytokines and chemokines by antigen presenting cells reflects signaling through both MyD88-dependent (TNF-α and IL-6) and -independent (RANTES) pathways. Recent studies using LPS-activated macrophages and iNOS-2 knockout mice suggest that iNOS activity and the production of reactive nitric oxide species contribute to the killing of intracellular *B mallei*.[98,227] These studies illustrate the importance of TLR4 signaling and reactive nitric oxide species in macrophage activation and clearance of intracellular *B mallei*. The role of other pattern-recognition receptors (eg, TLR2 and NOD-like receptors) in *B mallei* infection remains to be investigated.

Relatively little is understood regarding the role of T-cell subsets in controlling *B mallei* infections. Recent studies suggest that strong T-cell helper (Th)1-polarized responses will likely be required for protection against *B mallei* infection.[231,232] More research is needed in this area. Maximizing the effectiveness of CMI responses is predicted to limit the duration of *B mallei* infections and to reduce disease pathology, and understanding how innate and adaptive immune responses fail to result in complete bacterial clearance may provide clues for the design of effective vaccination strategies.

Vaccine Candidates

No human or veterinary glanders vaccine exists. From 1895 to 1928 numerous attempts to vaccinate horses and laboratory animals against glanders were unsuccessful. Vaccines were initially prepared by treating bacterial cells with urea or glycerin[7] or by drying the glanders bacilli.[225] For most chronically infected horses, experimental vaccination did not change the course of their illness. Experiments on protective immunity in horses have given ambiguous results.[1,7] Mendelson reported guarded postvaccination success in a youth with severe ocular and oro-nasal involvement.[54] Although attempts (with limited success) to develop a glanders vaccine were initiated more than 100 years ago, using modern approaches to identify virulence factors and studying the ways putative vaccines modulate the immune system may aid in developing an efficacious glanders vaccine.

Interest in glanders vaccine development has increased in recent years, mostly resulting from *B mallei's* biothreat potential. Several up-to-date reviews provide further details of glanders vaccine development.[233–235] The most desirable glanders vaccine will be a recombinant protein or a biochemically purified preparation that gives long-term sterile immunity when administered. As previously mentioned, since *B mallei* is an intracellular pathogen it is likely that both CMI and humoral responses will be critical in developing protective immunity. Activating both arms of adaptive immunity and ultimately achieving sterile immunity will be significant challenges in *B mallei* vaccine design. A better understanding of the correlates of vaccine-induced immunity is needed.

Killed Whole Cells

The initial attempts to protect mice against an aerosol-acquired infection with *B mallei* using either an irradiation-killed *B mallei* or heat-killed *B pseudomallei*

cellular vaccine resulted in an increased time to death, compared to controls, but spleens of survivors were not sterile.[236] A nonviable *B mallei* cellular vaccine failed to protect mice from a parenteral live challenge.[237] This vaccine stimulated a weak mixed Th1- and Th2-like immune response. This study suggested that nonviable *B mallei* cell preparations may not protect mice because of the failure inducing a strong Th1-like immune response. In a subsequent study, adding IL-12 to an irradiated *B mallei* vaccine preparation stimulated a Th1-like antibody response and induced an increase in splenocyte proliferation and IFN-γ production in comparison to mice vaccinated with killed *B mallei* alone.[231] Following an IP challenge with *B mallei*, increased survival was observed in mice vaccinated with both IL-12 and killed bacteria in comparison to control mice. Sterile immunity was not achieved, and the spleens of the vaccinated survivors were enlarged and heavily infected with *B mallei*. More recent studies by Sarkar-Tyson et al and Whitlock et al using heat killed *B mallei* and/or *B pseudomallei* are consistent with the findings of Amemiya et al, and indicate that TNF-α, IFN-γ, and B cells are necessary for an effective immune response against the organism.[226,231,238]

Live Attenuated Vaccines

Three attenuated strains of *B mallei*, a CPS (*wcbB*) mutant, a branched-chain amino acid auxotroph (ilvI) mutant, and a carboxy-terminal protease (*ctpA*) mutant have been evaluated as vaccines in a BALB/c mouse model of glanders.[77,90,131] The CPS and *ilvI* vaccine strains were delivered aerogenically, and mice were subsequently exposed to wild type *B mallei* via whole-body aerosols. Immunization with the CPS mutant resulted in a Th2-like antibody response that failed to protect mice (all animals died within 5 days of infection). In contrast, immunization with the *ilvI* mutant resulted in a Th1-biased immune response that conferred significant protection against lethal aerosol challenges. At 1 month postchallenge, 25% and 50% of the mice had survived high- and low-dose aerosol challenges, respectively. Analysis of bacterial loads in the organs of the surviving mice revealed high numbers (>10^5 cfu) of *B mallei* in the spleens of all of the *ilvI* mutant vaccinated mice. *B mallei* was also present in the livers and lungs of most of the surviving animals, suggesting the development of a chronic infection. A *B mallei ctpA* mutant, which was evaluated as a live attenuated vaccine in CD1 mice, provided partial protection against an IP challenge of wild type *B mallei*.[131] Whereas 75% of the *ctpA* mutant-vaccinated mice survived the 15-day postchallenge, all survivors displayed splenomegaly and high splenic loads of *B mallei*. A mixed Th1/Th2-like antibody response was noted. Based on these studies, it appears that live attenuated strains promoting Th1-like antibody responses may be useful as glanders vaccine candidates.

Protein Subunit Vaccines

Two studies by Whitlock et al describe the identification and testing of purified *B mallei* protein antigens as potential vaccine candidates.[125,239] Immunogenic antigens were identified from genomic screens of *B mallei* expressed proteins. In initial studies, the candidate protein antigens included LolC (ABC transporter protein), BimA (autotransporter protein), BopA (T3SS effector protein), and Hcp1 (T6SS-1 component).[239] IN immunization with purified recombinant LolC, BimA, BopA, and Hcp1 alone or as a quadrivalent mixture, administered with CpG 2395 and ISCOM adjuvants, provided BALB/c mice significant protection against an IN challenge of *B mallei*. While the recombinant proteins protected the mice from the initial acute infection, sterile immunity was not achieved. In a subsequent study, recombinant forms of five additional *B mallei* proteins and GroEL were evaluated for their protective capacity in BALB/c mice.[125,194] Three of these proteins and GroEL provided partial protein against an IN challenge of *B mallei*. Several of the highly immunogenic proteins (eg, LolC and Hcp1) evaluated in these studies are also considered promising components for melioidosis vaccine development.[105,124,239]

Polysaccharide-based Subunit Vaccines

B mallei isolates appear to be capable of expressing only a limited repertoire of structurally diverse CPS and LPS antigens.[36,93,97] The protective efficacy of LPS and CPS as vaccines against *B pseudomallei* infection was tested in BALB/c mice and partial protection was observed.[240,241] Active immunization studies using CPS or LPS for protection against *B mallei* have not been reported; however, it has been shown that CPS- and LPS-specific mAbs passively protect animals from challenge with *B mallei*.[97,104] In addition, the bactericidal or opsonophagocytic activities of various anti-LPS or anti-CPS mAbs appeared to correlate with their ability to passively protect mice.[97,104] Such findings confirm the protective capacity of these surface exposed antigens and support the rationale for developing CPS and OPS-based glanders vaccines. However, since carbohydrates such as CPS and OPS are T-independent antigens that would be poorly immunogenic if administered in purified form, methodologies for the preparation of CPS- and OPS-protein conjugates have been developed.[242–245] Immunization of mice with CPS- or OPS-based glycoconjugates resulted in the generation of high titer carbohydrate-specific antibody responses.

Depending on the adjuvant system used, Th1- or Th2-polarized antibody responses could be achieved. The protective capacity of these glycoconjugates in animal models of glanders has not been reported. Further development of these antigenically defined CPS- and OPS-based vaccine candidates is an active area of research.

Immunotherapies

In addition to passive immunization studies with PS-specific antibodies, activators of innate immune responses have been experimentally evaluated as potential immunotherapies for pre- or postexposure prophylaxis. BALB/c mice pre-treated with CpG-containing oligodeoxynucleotides (CpG 7909) that signal through TLR9 exhibited elevated levels of IL-6, IL-12, and IFN-γ following an aerosol challenge with *B mallei*, resulting in lower numbers of bacteria in lungs and spleen, and prolonged survival.[232] Similarly, mucosal administration of cationic liposome DNA complexes (CLDC), potent activators of innate immunity, to BALB/c mice before or shortly after bacterial challenge generated nearly complete protection from inhalational challenge with 100% lethal doses of *B mallei*.[86] Substantially reduced acute organ pathology was observed in CLDC-treated mice in comparison to controls. Protection was dependent on CLDC-mediated induction of IFN-γ responses in lung tissues and was partially dependent on the activation of natural killer cells. These findings suggest that preexposure or timely postexposure therapy with CpG or CLDC may help to protect individuals exposed to aerosolized *B mallei*.

Control and Decontamination

Historically, no vaccines were successful in protecting animals from glanders. Control and eradication of the disease has been dependent on eliminating infected horses and preventing infected horses from entering glanders-free stables. The greatest risk for glanders exposure to humans—outside of a biowarfare attack—is infected equids, particularly the asymptomatic horse. When glanders infection is considered as a differential diagnosis in countries with ongoing or completed eradication programs, local and state public health and veterinary authorities should be contacted immediately. Where human infection has occurred, potential exposure to infected equids should be investigated by a team approach involving patient care personnel, public health officials, and local veterinarians. Equids suspected as a possible human exposure source should be tested and, if positive, humanely destroyed in accordance with the local regulatory animal health authority. Facilities and transporters traced back to positive equine cases should be quarantined and disinfected in accordance with the local animal health authority. Stall bedding, feed, and manure in the vicinity of infected livestock should be burned.

In the event of deliberate release of *B mallei*, emergency response personnel entering a potentially heavily contaminated area should wear personal protective equipment, which includes Tyvek coveralls (DuPont USA, Wilmington, DE), gloves, and powered air purifying respirators. Decontamination procedures for the patient include the removal and containment of outer clothing. Such clothing should be regarded as contaminated or high risk and handled in accordance with local protocol. All waste should be managed in accordance with BSL-3 containment protocols. Patient showers are indicated, preferably in a facility for which decontamination and containment can be managed. The risk of acquiring infection from contaminated persons and their clothing is low.[246] Personal protective equipment may prevent infection in those potentially exposed, including emergency responders.

Environmental contamination will decline over time as a result of sunlight exposure and drying. Monitoring highly contaminated areas is indicated, however, and the advice of foreign animal disease experts should be sought. *B mallei* can remain viable in tap water for at least 1 month.[40] *B mallei* can be destroyed by heating to at least 55°C for 10 minutes, and by ultraviolet irradiation. It is susceptible to several disinfectants including 1% sodium hypochlorite, chlorine dioxide, 5% calcium hypochlorite, 70% ethanol, 5% Micro-Chem Plus (National Chemical Laboratories Inc, Philadelphia, PA), 2% glutaraldehyde, 1% formaldehyde, iodine, benzalkonium chloride, 1% potassium permanganate, 3% solution of alkali, and 3% sulfur-carbolic solution. Phenolic and mercuric chloride disinfectants are not recommended.[7,42,247,248]

Because human-to-human transmission has occurred nosocomially and with close personal contact, standard precautions are recommended. These precautions include use of disposable gloves, face shields, surgical masks, and—when appropriate—surgical gowns to protect mucous membranes and skin. Personnel, microbiological, and containment procedures for BSL-3 are advised in the laboratory. Appropriate barriers to direct skin contact with the organisms are mandatory at all times.[249,250] Family contacts should be advised of blood and body fluid precautions for patients recovering at home. Barriers protecting mucus membranes, cuts and sores, and potential skin abrasions from genital, oral, nasal, and other body fluids are recommended.

Many countries have import restrictions for equids. Veterinary health authorities may require testing within a few weeks of shipment and again at the place of disembarkation, as well as documentation of the animal's location in the exporting country for the 6 months before shipment.[23] Restrictions vary by country and glanders-free status under the International Animal Health Code. The most current information regarding import and export should be sought from the regional animal health authority.

SUMMARY

B mallei is designated as a Tier 1 select agent by the Centers for Disease Control and Prevention, since the organism is considered to present a high risk of deliberate misuse and could pose a severe threat to public health and safety. *B mallei* also is believed to be moderately easy to disseminate, and enhancements to current diagnostic capabilities and disease surveillance are required to diagnose the disease rapidly and accurately. Given the biothreat potential associated with *B mallei*, raising the clinical index of suspicion for glanders in humans is crucial. The rarity of recent human cases may make glanders a difficult diagnosis even in regions with exceptional medical facilities. As with many rare diseases, final diagnosis and appropriate treatment is often delayed, with sometimes disastrous results. Without a higher index of suspicion, diagnostic laboratories may not conduct tests appropriate to detect *B mallei*, as what happened in the eighth US laboratory-acquired infection in 2000.[56]

The genetic homology between *B mallei* and *B pseudomallei* may cause confusion in identifying the infectious agent, especially in endemic areas for *B pseudomallei*. However, once they are thoroughly assessed, new developments in nucleic acid-based PCR and DNA sequencing assays may improve the accuracy and speed of *B mallei* diagnosis. These developments include PCR assays targeting *B mallei*-specific sequences or single nucleotide polymorphisms in the bimA gene or in a unique conserved region of the *B mallei* and *B pseudomallei* genomes, and DNA sequence analyses that identify gene fragments and single polymorphisms in the 16S ribosomal RNA gene specific for *B mallei*. Effective treatments for glanders are available; however, due in part to the development of chronic infections, the disease remains difficult to treat. More research on treatments for *B mallei* is warranted. Considering an aerosol threat from a virulent strain, studies to distinguish the effectiveness of therapeutic agents for treating septicemic and pulmonary infections are indicated. The potential for prophylactic treatment regimens should be further investigated.

Significant progress has been made toward a better understanding of host immunity to *B mallei* infection. Effective innate immune responses are essential for controlling the early phase of the infection, and monocytes and macrophages are crucial for limiting dissemination of the organism. The role of adaptive immune responses in controlling *B mallei* infection requires more investigation, although it is evident that T cell responses will be important for vaccine-induced immunity. Efforts aimed at developing effective vaccine candidates for prevention of glanders are critical, and research is active in this area. Overcoming chronic infections and achieving the ultimate goal of sterile immunity will be challenging. Investigations focusing on determining correlates of vaccine-induced protection in both acute and chronic infection are needed.

Aerosol dissemination of *B mallei* would likely cause disease in humans, equids, goats, and possibly cats in the vicinity. Unintentional infection may first manifest in equids or humans. Thus, public health workers should team with animal health officials in a suspected outbreak to expedite identification and control of an event. Although a formal surveillance system for glanders does not exist in the United States, local and state veterinary and public health authorities would be among the first to recognize a potential outbreak regardless of intent. These agencies would then work with the US Department of Agriculture, the Centers for Disease Control and Prevention, the Department of Health and Human Services, and the Department of Defense to control and eradicate the disease.

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Chapter 9

MELIOIDOSIS

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INTRODUCTION

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SUMMARY

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INTRODUCTION

In 1911, Captain Alfred Whitmore and Dr CS Krishnaswami described a previously unrecognized disease that was prevalent among the ill-nourished and neglected inhabitants of Rangoon, Burma.[1] The new disease resembled glanders, a zoonotic disease of equines,[2] and the gram-negative bacillus they isolated post-mortem from tissue samples resembled the glanders bacillus, *Bacillus mallei*.[3] However, the new bacillus could be differentiated from *B mallei* by its motility, luxuriant growth on peptone agar, and wrinkled colony morphology, and was subsequently named *Bacillus pseudomallei*.[3,4] Whitmore's detailed account of the first 38 human cases of this disease demonstrated most were morphine injectors that died of septicemia with abscesses in multiple organs.[4] As a result, the disease became known as "Whitmore's disease" or "morphine injector's septicemia."[5,6] In 1921, Stanton and Fletcher reported an outbreak of a septicemic disease in a guinea pig colony at the Institute for Medical Research in Kuala Lumpur.[7] The infectious agent they isolated from diseased animals was indistinguishable from Whitmore's bacillus, and they coined the term "melioidosis" (a Greek term meaning glanders-like illness) to describe this new disease of the tropics.[7] Stanton and Fletcher subsequently published a classic monograph in 1932 describing their observations of melioidosis in humans and animals occurring in Burma, Malaya, French Indochina, and Ceylon over a number of years.[8]

Today, melioidosis is regarded as an emerging infectious disease and a potential bioterrorism threat.[9–11] The etiologic agent of melioidosis is present in water and soil in tropical and subtropical regions and is spread to humans through direct contact with the contaminated source. Clinical manifestations range from subclinical infection to overwhelming septicemia that resembles disseminated or localized, suppurative infection due to a variety of pathogens, resulting in the nickname "the remarkable imitator."[12] The majority of melioidosis cases have one or more identified risk factors, including diabetes, alcoholism, chronic renal disease, cystic fibrosis, and steroid abuse.[13] Interestingly, acquired immunodeficiency syndrome does not seem to be a major risk factor for melioidosis. Healthy individuals can also get the disease, especially if they work in muddy soil without good hand and foot protection.[14] Many animal species are susceptible to melioidosis, including sheep, goats, horses, swine, cattle, dogs, and cats.[15] Numerous review articles on melioidosis have been published since 1990.[11,13–30]

THE INFECTIOUS AGENT

The bacterium that causes melioidosis, now designated *Burkholderia pseudomallei*,[31] has undergone numerous name changes since its original classification as *Bacillus pseudomallei*, including *Bacterium whitmori, Bacillus whitmori*, *Pfeifferella whitmori*, *Pfeifferella pseudomallei*, *Actinobacillus pseudomallei*, *Lofflerella whitmori*, *Flavobacterium pseudomallei*, *Malleomyces pseudomallei*, and *Pseudomonas pseudomallei*. The non-sporulating, gram-negative bacillus is an environmental saprophyte found in surface waters and wet soils in endemic regions.[32–39] Individual cells are approximately 0.8 × 1.5 μm with a polar tuft of two to four flagella and may exhibit bipolar staining with a "safety pin" appearance.[40,41] *B pseudomallei* is metabolically versatile and can grow on numerous carbon sources.[31,42] Anaerobic growth is possible, but only in the presence of nitrate or arginine.[11] The microbe accumulates intracellular stores of poly-β-hydroxybutyric acid and can survive in distilled water for years.[10,43,44] The optimal survival temperature for *B pseudomallei* is between 24°C and 32°C, but it can grow at temperatures up to 42°C.[45,46] *B pseudomallei* demonstrate considerable interstrain and medium-dependent colony morphology.[47–49] The oxidase-positive organism can grow on a variety of microbial media, but Ashdown's selective medium is often used for isolating *B pseudomallei* from environmental and clinical specimens.[50] Two distinct colony phenotypes are commonly observed on this medium (Figure 9-1a), presumably due to the differential uptake of crystal violet and neutral red or to the differential production of ammonia and oxalic acid.[50,51] Most strains appear lavender after 2 to 3 days of incubation at 37°C, but some isolates appear deep purple (see Figure 9-1a). After 3 days at 37°C, the colonies often become dull and wrinkled (Figure 9-1b) and emit a distinctive sweet, earthy smell. Other selective media have also been used to isolate *B pseudomallei* from contaminated specimens.[52,53]

The complete genome sequence of *B pseudomallei* K96243, a strain isolated in 1996 from a 34-year-old diabetic patient in Khon Kaen, Thailand, was published in 2004.[54] The 7.25-megabase pair (Mb) genome was comprised of two circular replicons, termed chromosome 1 (4.07 Mb) and chromosome 2 (3.17 Mb). The G + C content of the genome is 68% and is predicted to encode 5,855 proteins. Chromosome 1 encoded a high proportion of core housekeeping functions (DNA replication, transcription, translation, amino acid and nucleotide metabolism, basic carbohydrate metabolism, and cofactor synthesis), while chromosome 2 encoded

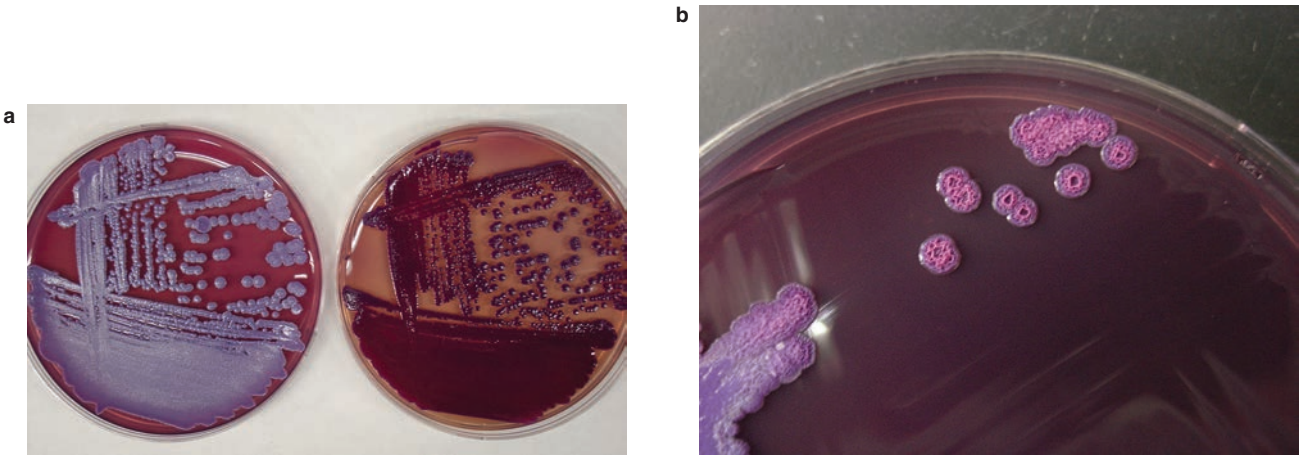


Figure 9-1. *Burkholderia pseudomallei* colony morphologies as demonstrated on Ashdown's selective medium[50] supplemented with 100 μg/mL streptomycin. Plates were incubated for 3 days at 37°C (**a**) and 5 days at 37°C (**b**).

a high proportion of accessory functions (adaptation to atypical conditions, osmotic protection, and secondary metabolism).[54] Plasmid-like replication genes and accessory genes on chromosome 2 suggest it may have been derived from a plasmid (or megaplasmid) that became an indispensable replicon by acquiring essential functions such as transfer ribonucleic acid genes, amino acid biosynthesis genes, and energy metabolism genes. There are 16 "genomic islands" in the *B pseudomallei* K96243 genome that appear to have been acquired through horizontal gene transfer.[54] Analysis of 11 additional *B pseudomallei* strains has led to the identification of at least 128 different genomic islands that are variably present among these strains.[55,56] Mobile genetic elements, such as prophages, insertion sequences, and integrated plasmids, account for most of the laterally acquired genomic sequences. Recent studies have shown that *B pseudomallei* strains exhibit significant genomic diversity and that much of the genetic heterogeneity is due to laterally acquired mobile genetic elements.[54,57–61] These genomic islands may provide strains that give them a metabolic or virulence advantage over strains that do not contain such sequences. Similarly, autonomously replicating plasmids are variably present in *B pseudomallei* isolates, but little is known about their biological significance.[30,62–64] Complete genome sequences of 13 *B pseudomallei* isolates (K96243, 1026b, 1106a, 1710b, 668, BPC006, MSHR146, MSHR305, MSHR511, NAU20B-16, NCTC13178, NCTC13179, MSHR520) and draft genome sequences of an additional 54 *B pseudomallei* isolates are available in GenBank, dramatically enhancing the amount and diversity of genome sequence data available for study of *B pseudomallei*.[65]

MILITARY RELEVANCE

Throughout the 20th century, melioidosis had an impact on the health of soldiers serving in Asia during times of war and peace.[66] Sporadic melioidosis infections occurred in US and Japanese soldiers during World War II (WWII),[41,67,68] and cases of recrudescent melioidosis in WWII veterans were also reported.[69,70] During the French Indochina War (1946–1954), there were at least 100 cases of melioidosis in French forces during their fight against the resistance movement led by the Viet Minh.[22,66] There were more than 300 cases of melioidosis in US soldiers during the Vietnam War[22] and additional cases that did not surface until years after the war's end, leading to the nickname "Vietnam Time Bomb."[71–73] Twenty-three melioidosis cases were reported in the Singapore Armed Forces from 1987 to 1994.[74] The infection rate in these relatively healthy Singapore Armed Forces was approximately fourfold the rate in the general population of Singapore, suggesting that close contact with the soil during military training may lead to an increased risk for melioidosis.

B pseudomallei is a Centers for Disease Control and Prevention Tier 1 select agent that must be handled in biosafety level 3 laboratories.[9] Biosafety level 3 facilities incorporate specialized negative-air pressure ventilation systems and well-defined biosafety containment equipment and protocols to study agents that can be transmitted through the air and cause potentially lethal infection. Tier 1 agents present the greatest risk of deliberate misuse and pose a severe threat to public health and safety. *B pseudomallei* was studied by the United States, the former Soviet Union, and possibly Egypt as a potential biological warfare agent, but was

never used in this capacity.[75–77] On the other hand, *B mallei* was used as a biological warfare agent several times in the past, including during the American Civil War, World War I, WWII, and in Afghanistan between 1982 and 1984.[2,76,78,79] The usefulness of *B pseudomallei* as a biological warfare agent is currently unknown, but the ease of acquiring strains from the environment, the ability to genetically manipulate the agent to be multiply antibiotic resistant, and the lack of a melioidosis vaccine make this possibility a serious concern.

THE DISEASE

Epidemiology

Melioidosis cases are most commonly reported from countries located between 20°N and 20°S in latitude, with the greatest concentration in Vietnam, Cambodia, Laos, Thailand, Malaysia, Singapore, and northern Australia.[11,13,23] The disease has also been observed in the South Pacific, Africa, India, and the Middle East.[80–83] In addition, sporadic cases of melioidosis have occurred in the Western Hemisphere in Aruba, Brazil, Mexico, Panama, Ecuador, Haiti, Peru, and Guyana.[11,13,23,84,85] In endemic regions, the disease occurs in humans, sheep, goats, horses, swine, cattle, dogs, cats, deer, camels, monkeys, zebras, kangaroos, koalas, birds, and crocodiles.[15,27,86–88] Melioidosis cases that occur in temperate regions are typically acquired in endemic areas. Human cases can often be attributed to recent travel to such areas.[21,89–92] For example, the first case report of cerebral melioidosis in the United States was recently described in a individual that had immigrated from Cambodia and frequently visited his home country.[93] A case of neurological melioidosis was reported in a pigtail macaque that was imported from Indonesia to the United States for research purposes.[94] In addition, *B pseudomallei* infections have been described in pet green iguanas in California and, based on the multilocus sequence typing of the isolates in these cases, are believed to have originated in Central America.[95] With the exception of a single case of melioidosis that occurred in Arizona in 2008, no *B pseudomallei* infections have been documented in US patients without a history of prior travel to an endemic region.[96,97] While the source of the Arizona isolate could not be determined despite extensive investigation, molecular analysis indicated that it was consistent with Southeast Asian origin.[97,98]

Pathogenesis

Several animal models of melioidosis have been developed to study pathogenesis, virulence factors, and efficacy of antibiotics and vaccines.[99–107] In general, hamsters and ferrets are highly susceptible to experimental melioidosis, while rats, pigs, and rhesus monkeys are relatively resistant. Infant rats can be made more susceptible to infection by intraperitoneal injection of streptozotocin, a compound that induces diabetes.[103,108] The LD_{50} (amount necessary to kill 50% of the subject population) of *B pseudomallei* for non-diabetic infant rats is greater than 10^8 bacteria, while the LD_{50} in streptozotocin-induced diabetic infant rats is approximately 10^4 bacteria. Mice and guinea pigs exhibit intermediate susceptibility to experimental infection with *B pseudomallei*, but the LD_{50} for mice varies widely depending on the route of infection, mouse strain, and bacterial strain.[101,102,105,109] Recent reviews describe in detail the various mouse models of melioidosis that have been developed and the advantages and disadvantages of each.[110,111]

Due to fears that *B pseudomallei* might be used as a biological weapon, basic research on this pathogen has progressed rapidly over the past 10 years. The identification of virulence factors has been facilitated by the availability of genomic sequence data[54] and the existence of a nonpathogenic *B pseudomallei*-like species designated *B thailandensis*.[112–114] *B pseudomallei* and *B thailandensis* strains are genetically and immunologically similar to one another, but *B thailandensis* is less virulent in animal models of infection and has only rarely been reported to cause disease in humans.[115] Genetic determinants that confer enhanced virulence in *B pseudomallei* relative to *B thailandensis* have been identified by comparative analysis of genomic DNA from these species.[58,116,117] Table 9-1 provides a brief description of *B pseudomallei* virulence factors identified to date, their mechanisms of action, and their relative importance in animal models of melioidosis.

B pseudomallei is a facultative intracellular pathogen whose ability to survive and replicate in phagocytic and nonphagocytic cell lines has been well documented.[118–123] The organism possesses a variety of mechanisms to adapt to the host environment and harbors an array of specialized secretory systems, which are required within this niche.[124–128] Although relatively little is known about the initial interactions of *B pseudomallei* with host cells, recent studies have identified a number of adhesins that mediate adherence of the organism to eukaryotic cells.[129–133] Following internalization, *B pseudomallei* rapidly escapes from endocytic vacuoles and enters into the host cell cytosol, where it can replicate to high numbers, polymerize actin, and induce host cell fusion. These processes are believed

to facilitate intracellular spread and multinucleated giant cell formation. Type III and type VI secretions systems have been shown to play key roles in endosomal escape and multinucleated giant cell formation, respectively.[124,134] The effector proteins delivered by these systems are predicted to modulate critical host proteins and pathways involved in cytoskeleton rearrangement, cell signaling, and cell death, thereby enabling pathogen survival and proliferation within a host.[17] It has been postulated that after the initial phase of infection, *B pseudomallei* can persist in a dormant stage in macrophages for months or years.[123] Melioidosis has the potential for a long latency period and *B pseudomallei*'s intracellular persistence could provide a mechanism by which this occurs. Intracellular survival and cell-to-cell spread may also provide *B pseudomallei* protection from the humoral immune response.

Clinical Disease

Melioidosis is a tropical bacterial disease with primary endemic foci in southeast Asia, northern Australia, south Asia, and China. Hyperendemic areas for melioidosis include northern Australia and northeast Thailand, where the disease incidence peaks in the rainy season. The routes of infection include percutaneous inoculation, inhalation, or ingestion of contaminated food or water. Although percutaneous inoculation is the most common route of infection, heavy rainfall is associated with pneumonia and more severe disease and may represent a shift from percutaneous inoculation to inhalation as the primary mode of infection.[135] In hyperendemic areas, *B pseudomallei* causes a substantial burden of infectious disease. For example, at the Sappasit Prasong Hospital in Ubon Ratchatani, northeast Thailand, which serves a rural community of rice farmers and their families, nearly 20% of all community-acquired bacteremia is due to *B pseudomallei*.[136] Likewise, melioidosis is the most common cause of fatal community-acquired bacteremic pneumonia at the Royal Darwin Hospital in the Northern Territory of Australia.[137]

Cases of human-to-human transmission of *B pseudomallei*, although very rare, have been documented.[138,139] The incubation period (time between exposure and appearance of clinical symptoms) varies, as infectious dose, route of infection, *B pseudomallei* strain characteristics, and host risk factors are all believed to play an important role. One study that looked at the incubation period after inoculation exposures in Darwin, Australia, revealed a mean incubation period of 9 days, with a range of 1 to 21 days.[140] Although serologic studies suggest that most infections with *B pseudomallei* are asymptomatic,[141] individuals with risk factors such as diabetes mellitus, alcoholism, cirrhosis, thalassemia, or other immunosuppressed states are at an increased risk of developing symptomatic infection. Other melioidosis-associated risk factors include chronic lung disease, kava consumption, and cystic fibrosis. Diabetes appears to be the most important of all the known risk factors, as up to 50% of patients with melioidosis have diabetes mellitus.[27]

Melioidosis, which presents as a febrile illness, has an unusually broad range of clinical presentations. The diversity of infectious presentations includes acute localized suppurative soft tissue infections, acute pulmonary infections, acute fulminant septicemia, and chronic localized infections. Clinical disease with *B pseudomallei* is generally caused by hematogenous spread of bacteria and seeding to various organs within the host.[27] The Infectious Disease Association of Thailand, the country with the largest number of reported cases (2,000–3,000 per year), divided 345 cases into the following categories: (*a*) disseminated septicemia, 45% of the cases with 87% mortality; (*b*) nondisseminated septicemia, 12% of the cases with 17% mortality; (*c*) localized septicemia, 42% of the cases with 9% mortality; and (*d*) transient bacteremia, 0.3% of cases.[142,143]

Melioidosis is characterized by abscess formation, and the majority of patients with melioidosis are bacteremic. The most commonly involved organ is the lung. The nidus of infection is either a primary pneumonia or lung abscess, or the infection results from hematogenous seeding of the lung from bacteremia (Figures 9-2 and 9-3). For example, of the 540 cases of melioidosis analyzed in the 20-year Darwin Prospective Melioidosis Study, pneumonia was the most common primary

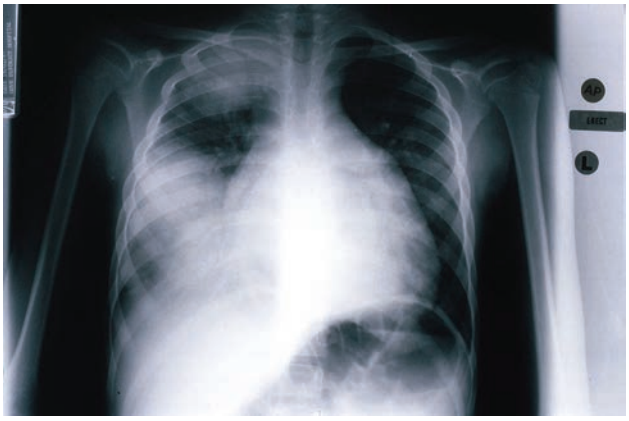


Figure 9-2. Chest radiograph demonstrating a severe multilobar pneumonia.
Photograph courtesy of Bart Currie, MD, Royal Darwin Hospital, Australia.

TABLE 9-1

CANDIDATE VIRULENCE FACTORS OF *BURKHOLDERIA PSEUDOMALLEI*

Factor	Description
Capsular polysaccharide	CPS is a 200-kDa 1,3-linked 2-*O*-acetyl-6-deoxy-β-D-*manno*-heptan.[1] CPS mutants are highly attenuated in hamsters and mice.[2,3] The CPS may contribute to survival in serum by reducing complement factor C3b deposition.[4,5]
Type III secretion system	*B pseudomallei* harbors three distinct TTSS loci, termed TTSS1, TTSS2, and TTSS3.[6] The TTSS1 and TTSS2 loci are similar to TTSS genes of the plant pathogen *Ralstonia solanacearum* and are not necessary for virulence in hamsters.[6] The TTSS3 locus is similar to the TTSS in *Salmonella* and *Shigella*[7] and is required for full virulence of *B pseudomallei* in both hamsters and mice.[6,8] The effector proteins of TTSS3 facilitate the invasion of epithelial cells and escape from endocytic vesicles.[7,9]
Type VI secretion system	*B pseudomallei* harbors six distinct T6SS loci designated T6SS-1, T6SS-2, T6SS-3, T6SS-4, T6SS-5, and T6SS-6.[10] Of the six systems, only T6SS-1 has been shown to be necessary for virulence in animal models of melioidosis. This system is expressed following uptake by murine macrophages (RAW264.7 cells) and is required for optimal intracellular growth, actin-based motility and multinucleated giant cell formation.[11,12,13]
Transcriptional regulators and two-component regulatory systems	*B pseudomallei* harbors numerous transcriptional regulators and two component regulatory systems. *BspR* (TetR family regulator), *bsaN*, and *bprC* (AraC family regulators) are involved in a complex regulatory cascade that ultimately controls TTSS3 and T6SS-1 gene expression.[14,15] *B pseudomallei bspR*, *bsaN*, and *bprC* mutants exhibited significantly attenuated virulence in mice.[12,14,15] The VirAG two-component regulatory system is known to positively regulate T6SS-1 gene expression, and mutations in VirAG render the organism avirulent in mice.[11,12,14]
Quorum sensing	*B pseuβdomallei* encodes three *luxI* homologues that produce at least three quorum-sensing molecules, including *N*-octanoyl-homoserine lactone (C8-HSL),[16,17] *N*-decanoyl-homoserine lactone (C10-HSL),[16,18] and *N*-(3-hydroxyoctanoyl)-L-homoserine lactone (3-hydroxy-C8-HSL).[16] It also has five *luxR* homologs to sense these signals. Mutations in all of the *luxI* and *luxR* homologues result in strains with decreased virulence in hamsters and mice,[16,18] but the virulence-associated genes regulated by this complex quorum-sensing system are still under investigation.
Lipopolysaccharide O-antigen	An unbranched heteropolymer consisting of disaccharide repeats having the structure -3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranose-(1-, in which the 6-deoxy-α-L-talopyranose residues posses 2-*O*-acetyl or 2-*O*-methyl and 4-*O*-acetyl modifications.[19,20,21] LPS O-antigen mutants are attenuated in hamsters, guinea pigs, and infant diabetic rats and are killed by serum.[22] This factor promotes survival in serum by preventing killing by the alternative pathway of complement. Levels of anti-LPS O-antigen antibodies are significantly higher in patients who survive than in those who die.[23]
Flagellin	A surface-associated 43-kDa protein that is required for motility.[24,25] Flagellin mutants are attenuated in mice,[26] but not in hamsters or infant diabetic rats.[25] Passive exposure studies demonstrated that flagellin-specific antiserum was capable of protecting infant diabetic rats from challenge with *B pseudomallei*.[24]
Type II secretion	Required for the secretion of several exoproducts, including protease, lipase, and phospholipase C.[27] The products secreted by this pathway appear to play a minor role in *B pseudomallei* pathogenesis.[28]
Type IV pili	*B pseudomallei* K96243 encodes four complete type IV pilin clusters.[29] A mutation in *pilA*, a gene encoding a type IVA pilin subunit, resulted in a strain exhibiting decreased attachment to cultured respiratory cell lines relative to wild type. The *pilA* mutant was not attenuated in mice by the intraperitoneal challenge route, but was slightly attenuated by the intranasal challenge route.[30]
Biofilm formation	The extracellular slime matrix produced by *B pseudomallei* appears to be polysaccharide in nature, but the exact structure is unknown.[31] Biofilm mutants were not attenuated in the mouse model of melioidosis, suggesting that the biofilm plays a relatively minor role, if any, in virulence.[31]
Malleobactin	A water-soluble siderophore of the hydroxamate class.[32] The compound is capable of scavenging iron from both lactoferrin and transferrin in vitro.[33] The genes encoding malleobactin biosynthesis and transport were recently identified, but malleobactin mutants were not tested in animal models of melioidosis.[34]
Rhamnolipid	A 762-Da glycolipid with the structure 2-*O*-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxytetradecanoyl-β-hydroxytetradecanoate (Rha-Rha-C14-C14).[35] Rhamnolipid-treated cell lines exhibit profound morphological alterations, but the role of this glycolipid in virulence remains unknown.[36]

(**Table 9-1** *continues*)

Table 9-1 continued

Exopolysaccharide	A linear, unbranched polymer of repeating tetrasaccharide units composed of D-galactose and 3-deoxy-D-*manno*-octulosonicacid (KDO), with the following structure: -3)-2-*O*-Ac-β-D-Gal*p*-(1-4)-α-D-Gal*p*-(1-3)-β-D-Gal*p*-(1-5)-β-D-KDO*p*-(2-.[37–39] EPS is not produced by the closely related nonpathogenic species *B thailandensis*, suggesting that it may be a virulence determinant of *B pseudomallei*. EPS is probably produced during infection because sera from melioidosis patients contain IgG and IgM antibodies to EPS.[38,40]
Endotoxin	The lipid A portion of *B pseudomallei* LPS contains amide-linked 3-hydroxyhexadecanoic acids, which are longer than the fatty acid chains of enterobacterial LPS.[41] The endotoxic activity of *B pseudomallei* LPS was 10- to 100-fold weaker than enterobacterial LPS in pyrogenic activity in rabbits, lethal toxicity in GalN-sensitized mice, and macrophage activation assays. However, the mitogenic activity of *B pseudomallei* LPS was much higher than enterobacterial LPS.[41] The LD_{50} of purified *B pseudomallei* LPS in hamsters was 1,000 mg.[42]
Actin-based motility	Once *B pseudomallei* gains access to the host cell cytoplasm, it can replicate and exploit actin-based motility for cell-to-cell spread and evasion of the humoral immune response.[43–45] The autotransported protein *bimA* is located at the pole of the bacterial cell and is responsible for the formation of actin tails.[44] It is currently unknown if actin-based motility is required for virulence in animal models of melioidosis.
Autotransporters and adhesins	*B pseudomallei* harbors 11 autotransporter analogs, including *bimA*, and two ATs (*boaA* and *boaB*) with roles in bacterial adhesion to epithelial cells, eight ATs (BpaA-F; BcaA-B) that contribute to adherence to and efficient invasion of A549 cells.[46–48] *BpaC* and *bcaA* have been implicated in virulence in BALB/c mice, since mutants in these ATs displayed defects in dissemination to the liver or spleen, respectively.[47] A *B pseudomallei bbfA* (also known as *bpaF*) mutant demonstrated a moderate attenuation in a murine model of melioidosis.[48]
Exotoxins	There have been several reports in the literature about *B pseudomallei* exotoxins,[49–53] but the genes encoding these exotoxins have not been identified and no defined exotoxin mutants have been constructed. The role of exotoxins as *B pseudomallei* virulence factors is highly controversial and there appears to be no correlation between in vitro cytotoxicity and in vivo virulence.[42,54] In fact, the K96243 genome sequence does not encode any homologues of known major toxins produced by other pathogenic bacteria.[29]
Intracellular toxin	*Burkholderia* lethal factor 1 (BLF1; BPSL1549) is structurally related to cytotoxic necrotizing factor 1 and has been shown to specifically deamidate Gln^{339} of eukaryotic initiation factor 4A (a translation initiation factor) leading to inhibition of protein synthesis.[55,56] Purified recombinant BLF1 was toxic to mice (via intraperitoneal injection), J774 macrophages, but not 3T3 cells.[55,56] A *B pseudomallei bfl1* mutant was significantly attenuated in mice and exhibited a 100-fold higher median lethal dose in comparison to the wild type strain.[55,56]

CPS: capsular polysaccharide; EPS: exopolysaccharide; Gln: glutamine; IgG: immunoglobulin G; IgM: immunoglobulin M; LPS: lipopolysaccharide; T6SS: type VI secretion system; TTSS: type III secretion system

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(**Table 9-1** *continues*)

Table 9-1 *continued*

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clinical presentation, occurring in 278 (51%) of the patients.[144] Patients with acute pulmonary melioidosis present with cough, fever, sputum production, and respiratory distress, and can present with or without shock. Chronic pulmonary melioidosis mimics tuberculosis, with side effects including purulent sputum production, cough, hemoptysis, and night sweats.

Patients with the acute septic form of melioidosis present characteristically with a short history of fever and no clinical evidence of focal infection. Most patients are profoundly ill with signs of sepsis. Septic shock may appear on presentation. In the Darwin Prospective Melioidosis Study, 298 (55%) of patients were bacteremic on presentation to the hospital. Septic shock, usually occurring on, or within 24 hours of, admission to the hospital, was associated with 50% mortality, while bacteremia without septic shock was associated with a 7%

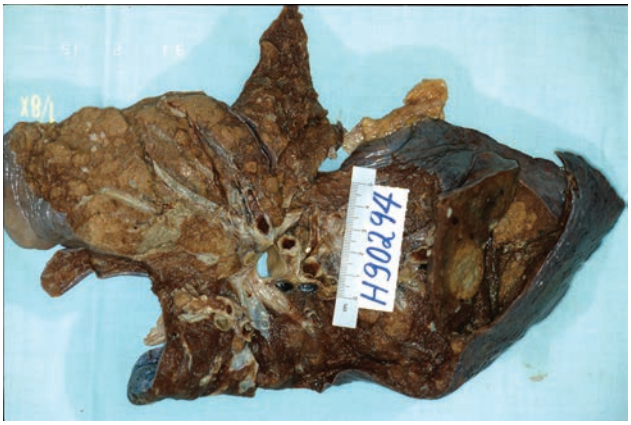


Figure 9-3. Autopsy specimen demonstrating extensive pulmonary involvement with abscess formation due to *B pseudomallei*.
Photograph courtesy of Bart Currie, MD, Royal Darwin Hospital, Australia.

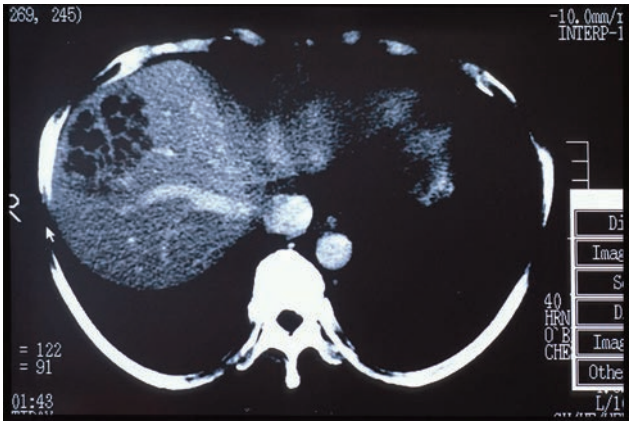


Figure 9-5. Computed tomography scan showing multiloculated liver abscess.
Photograph courtesy of Bart Currie, MD, Royal Darwin Hospital, Australia.

mortality.[144] Hematogenous seeding and abscess formation can occur in any organ (Figure 9-4); however, liver, spleen, skeletal muscle, prostate, and kidney are the most common abscess sites (Figures 9-5 and 9-6).[27]

Less common presentations of melioidosis include uncomplicated infections of the skin (Figure 9-7), subcutaneous tissues, or the eye. Corneal ulcerations resulting from trauma, which become secondarily infected with *B pseudomallei*, are rapidly destructive.[145] Septic arthritis and osteomyelitis (Figure 9-8) have also been described, but cellulitis appears to be rare. In a prospective study of more than 2,000 patients with melioidosis in Thailand, primary meningitis or endocarditis was not observed, but meningitis secondary to cerebral abscess rupture and mycotic aneurysms was seen.[27] Other unusual melioidosis presentations include mediastinal masses, pericardial fluid collections, and adrenal abscesses. The clinical presentation of melioidosis also varies among different regions. In Thailand, 30% of the melioidosis cases in children present as acute suppurative parotitis.[136] These children present with fever, pain, and swelling over the parotid (salivary) gland without other evidence of underlying predisposing conditions. In 10% of the cases, the swelling is bilateral.[27] Although acute suppurative parotitis is unusual in Australia, approximately 4% of the melioidosis cases there present as brainstem encephalitis with peripheral motor



Figure 9-4. Pustules with an erythematous base due to septicemic melioidosis.
Photograph courtesy of Bart Currie, MD, Royal Darwin Hospital, Australia.

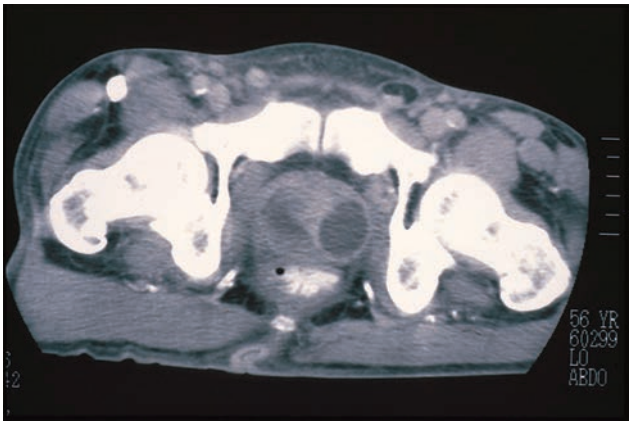


Figure 9-6. Computer tomography scan showing prostatic abscess.
Photograph courtesy of Bart Currie, MD, Royal Darwin Hospital, Australia.



Figure 9-7. Skin lesions associated with melioidosis on the lower extremity.
Photograph courtesy of Bart Currie, MD, Royal Darwin Hospital, Australia.

weakness or flaccid paraparesis. Features associated with this presentation include limb weakness, cerebellar signs, and cranial nerve palsies. Patients with this syndrome usually have an initial normal state of consciousness. Multiple focal *B pseudomallei* micro abscesses in the brainstem and spinal cord are probably responsible for this syndrome.[27]

Although acute infections in individuals with predisposing risk factors are the most common, latent infection with reactivation, resulting in an illness that can resemble tuberculosis, also occurs with melioidosis. During the Vietnam War, large numbers of Western soldiers were exposed to *B pseudomallei* through inhalation, contaminated wounds, or burns. A serologic survey of US military personnel demonstrated that mild or unapparent infection was common, and estimated that 225,000 people with subclinical infection were potentially at risk for reactivation.[146] Fortunately, the number of cases of reactivation melioidosis in these individuals has remained rare compared to the number of individuals exposed. Long latency periods between exposure and development of melioidosis in nonendemic regions have been reported.[70] Recently, a case of cutaneous melioidosis in a man taken prisoner by the Japanese during World War II was described. This man is presumed to have had reactivated melioidosis 62 years after exposure, as he had not returned to an area of melioidosis endemicity after being imprisoned in northwest Thailand.[69]



Figure 9-8. Chronic osteomyelitis of the lower extremity due to melioidosis.
Photograph courtesy of Bart Currie, MD, Royal Darwin Hospital, Australia.

Diagnosis

Because of its protean clinical manifestations, the diagnosis of melioidosis depends on the isolation and identification of *B pseudomallei* from clinical specimens. Melioidosis should be suspected in any severely ill, febrile patient with an associated risk factor who has been in an endemic area. *B pseudomallei* can grow on most routine laboratory media and can be isolated from normally sterile sites, such as blood, by standard techniques.[23] The organism is usually detected in blood culture within 48 hours. Isolator centrifugation blood culture systems result in quicker detection times, but are less sensitive compared to conventional broth-based blood culture.[147] Ashdown's medium, a crystal violet and gentamicin-containing medium that permits selective growth of *B pseudomallei* (see Figure 9-1), has been used to significantly increase the frequency of recovery of *B pseudomallei* from the rectum, wounds, and sputum compared to recovery on blood and MacConkey agars.[50] Patients with suspected melioidosis should submit blood, sputum, urine, and abscess fluid, as well as throat wound and rectal swabs for culture.

B pseudomallei is intrinsically resistant to aminoglycosides and polymyxins.[148,149] This unusual antibiotic profile (gentamicin and colistin resistance, but amoxicillin-clavulanate susceptibility) in an oxidase-positive, gram-negative bacillus is helpful for identifying *B pseudomallei* in the microbiology laboratory. Commercially available kits for bacterial identification, such as the API 20NE (bioMérieux, Marcy l'Etoile, France), have been reported to reliably confirm the identity of *B pseudomallei*,[49] although other investigators have reported mixed results.[150] The Vitek 1 (bioMérieux) has also been found to be highly sensitive, having identified 99% of the 103 *B pseudomallei* isolates tested.[151]

However, in this same study, the Vitek 2 (bioMérieux) identified only 19% of these same isolates.[151] Other studies continue to document difficulties in identifying *B pseudomallei* with the Vitek 2.[152,153] It has also been recently reported that *B pseudomallei* specimens from infections that occurred in exotic locations, such as Malaysian Borneo, were misidentified as *B cepacia* by the Vitek 2.[154]

Serologic testing alone is not a reliable method of diagnosis. An indirect hemagglutination test and other serologic tests may produce false negatives in patients with sepsis, as well as false positives due to a high prevalence of antibodies to *B pseudomallei* in healthy individuals from endemic areas.[143] A recently published paper from Australia proposed a highly sensitive *B pseudomallei* identification algorithm that makes use of screening tests (Gram stain, oxidase test, gentamicin, and polymyxin susceptibility testing) combined with monoclonal antibody agglutination testing and gas-liquid chromatography analysis of bacterial fatty acid methyl esters.[155] Various polymerase-chain-reaction–based identification techniques have also been developed to aid in the identification of *B pseudomallei*.[156,157] A recent comparison of the sensitivities and specificities of seven different real-time TaqMan (Life Technologies, Grand Island, New York) polymerase chain reaction assays for detecting *B pseudomallei* demonstrated that an assay targeting the type III secretion system (TTS1-ofr2) performed the best at detecting *B pseudomallei* directly from clinical samples.[158]

Treatment

Asymptomatic carriage of *B pseudomallei* appears to be a very rare event[159]; therefore, the isolation of *B pseudomallei* from a clinical specimen indicates that treatment is required. Melioidosis requires prolonged antibiotic therapy to cure the infection and prevent relapse. Melioidosis cases should be treated with initial intensive therapy (at least 2 weeks of intravenous [IV] therapy) followed by oral eradication therapy for a minimum of 3 months. Cases presenting with localized mild disease can be treated with oral eradication therapy without initial parenteral treatment. The choice of therapy for treating melioidosis is complicated because *B pseudomallei* is resistant to many antibiotics, including penicillin, ampicillin, aminoglycosides, first- and second-generation cephalosporins, and colistin.[160,161] *B pseudomallei* is also relatively insensitive to quinolones and macrolides[162]; therefore, therapeutic options are limited.

The first study demonstrating the effectiveness of ceftazidime for severe melioidosis was published in 1989. In this study, ceftazidime treatment (120 mg/kg/day) was associated with a reduction of overall mortality from 74% to 37% ($P = 0.009$) when compared to "conventional therapy" with chloramphenicol (100 mg/kg/day), doxycycline (4 mg/kg/day), trimethoprim (TMP; 10 mg/kg/day), and sulfamethoxazole (SMX; 50 mg/kg/day).[163] In 1992, a second randomized clinical trial of treatment of severe melioidosis conducted in Thailand also demonstrated a substantial reduction in mortality when ceftazidime plus TMP-SMX was used, as compared to the four-drug conventional therapy.[164]

In 1999, a comparative treatment trial in Thailand found that imipenem/cilastatin was as effective as ceftazidime for the treatment of severe melioidosis. Although there was no difference in mortality, fewer treatment failures were observed in the patients given imipenem/cilastatin as compared to the ceftazidime group.[165] Therefore, initial intensive therapy for melioidosis should consist of high doses of ceftazidime (50 mg/kg, up to 2 g IV every 6 hours), imipenem/cilastatin (25 mg/kg, up to 1 g IV every 6 hours), or meropenem (25 mg/kg, up to 1 g IV every 8 hours) combined with TMP-SMX (320 mg/1,600 mg IV or by mouth every 12 hours) for patients with severe infection involving the brain, prostate, or other privileged site, for at least 14 days.[166] Critically ill patients with extensive pulmonary disease, organ abscesses, osteomyelitis, septic arthritis, or neurological melioidosis require longer intensive IV therapy (4 weeks or even longer).

The benefit of adding TMP-SMX to the initial antimicrobial regimen is supported by animal data and expert opinion.[26] However, a 2005 paper from Thailand, which described two randomized controlled trials comparing ceftazidime alone versus ceftazidime combined with TMP-SMX for severe melioidosis, failed to demonstrate a mortality benefit associated with the addition of TMP-SMX, although the dose of TMP-SMX used in this study appears to be lower than that used in Australia.[167] Nonetheless, patients in the Royal Darwin Hospital in the Northern Territory of Australia with severe melioidosis involving the brain, bone, prostate or other sequestered site, or severe pulmonary disease are treated with meropenem and TMP-SMX (Bart Currie, MD, Royal Darwin Hospital, Australia, written communication, April 2013). Meropenem is used rather than imipenem/cilastatin because it has fewer neurological side effects.[162]

The median time to resolution of fever is 9 days, but patients with large abscesses or empyema often have fluctuating fevers lasting a month or more. In a 10-year prospective review of 252 melioidosis cases in Australia, internal organ abscesses were common, with the largest majority found in the prostate. Although other internal collections frequently resolve with medical therapy, prostatic abscesses usually require drainage to prevent treatment failures.[137]

Adjunctive therapy with recombinant granulocyte colony-stimulating factor (G-CSF) is routinely used for melioidosis patients with septic shock in the Northern Territory of Australia. A retrospective review of mortality rates before and after the addition of G-CSF therapy at the Royal Darwin Hospital was recently published. In this study, the introduction of G-CSF as adjunctive therapy for patients with septic shock was associated with a decrease in mortality from 95% to 10%.[168] However, a randomized controlled clinical trial of G-CSF for treating melioidosis sepsis in Thailand failed to demonstrate a significant mortality benefit to the group that received it in addition to ceftazidime. The authors pointed out that the resource-constrained environment where the trial took place (limited ventilator and inotropic support, no invasive monitoring, no dialysis) may have introduced confounding variables.[169]

After initial intensive therapy, oral maintenance therapy is given for another 12 to 20 weeks to prevent disease relapse.[13] Oral maintenance therapy traditionally consists of chloramphenicol (40 mg/kg/day), doxycycline (4 mg/kg/day), and TMP-SMX (10 mg/50 mg/kg/day).[170] However, this combination frequently causes side effects that result in problems with compliance. Some experts recommend high-dose TMP-SMX (8 mg/40 mg/kg, up to 320/1,600 mg, by mouth twice daily) combined with doxycycline.[142] The combination of TMP-SMX with doxycycline was recently shown to be as effective and better tolerated than the conventional four-drug regimen (chloramphenicol, doxycycline, and TMP-SMX) for maintenance therapy in an open-labeled randomized trial conducted in Thailand.[171] In this study, failure to complete at least 12 weeks of maintenance therapy was the most important determinate of relapse. In the Northern Territory of Australia, TMP-SMX (8 mg/40 mg/kg) given every 12 hours is used as monotherapy for maintenance for at least 3 to 6 months, with a low relapse rate (1 failure in fewer than 60 patients).[13,137] It should be noted, however, that improvements in rates of relapse, from 6.4% (prior to September 2003) to 1.2% (after September 2003) in the Darwin Prospective Melioidosis Study have been attributed to the improved used of efficacious antimicrobials as well as a lengthened IV treatment phase for complex cases.[172] A recent, multicenter, double-blind, randomized placebo-controlled trial comparing the efficacy of 20 weeks of TMP-SMX plus placebo to TMP-SMX plus doxycycline for oral maintenance therapy was recently published. This trial, which enrolled 626 patients from five hospitals in northeast Thailand, demonstrated that TMP-SMX was noninferior to TMP-SMX plus doxycycline for the oral phase of melioidosis treatment. Adverse drug reactions were less common in the TMP-STX group than in the TMP-SMX plus doxycycline group, suggesting that TMP-STX monotherapy is preferred on the basis of safety and patient tolerance.[173]

Although evidence suggests that it is associated with a higher rate of relapse, amoxicillin-clavulanate can be used for oral maintenance therapy in individuals with a sulfonamide allergy or in pregnant patients. The recommended dose of oral amoxicillin-clavulanate is 20 mg/5 mg/kg, three times a day—a dose of amoxicillin-clavulanate that is higher than usually prescribed.[174]

Quinolone antibiotics are not recommended as therapy for eradicating *B pseudomallei*. Ciprofloxacin and ofloxacin were found inferior, with a failure rate of 29%, when compared to a 20-week course of maintenance therapy consisting of amoxicillin-clavulanate or the combination of chloramphenicol, doxycycline, and TMP-SMX.[175] Another study also found that the combination of ciprofloxacin plus azithromycin was associated with an unacceptably high rate of relapse.[176]

Prevention

Several experimental melioidosis vaccines have been tested in rodent models of infection, including live attenuated vaccines, heterologous vaccines, acellular vaccines, and subunit vaccines.[177–180] Variability in vaccination protocols, routes of challenge, and animal models make it difficult to directly compare the experimental melioidosis vaccine studies published. In general, most vaccine candidates provided significant protection compared to unvaccinated controls, but none resulted in 100% protection and sterilizing immunity. Several recent comprehensive reviews thoroughly describe the melioidosis vaccine candidates that have been developed to date.[178–180]

Live attenuated vaccines have been shown to be immunogenic and protective against a variety of facultative intracellular pathogens, including *Mycobacterium tuberculosis*, *Shigella*, *Salmonella*, *Yersinia*, *Listeria monocytogenes*, *Francisella tularensis*, and *Brucella melitensis*.[181–185] *B pseudomallei* purine auxotrophic mutants generated by ultraviolet and chemical mutagenesis were highly attenuated in mice and provided significant protection against subsequent challenge with virulent strains.[186,187] Unfortunately, the molecular nature of the purine-dependent mutations in these strains was unknown, and the possibility of reversion to wild-type could not be eliminated. A *B pseudomallei* temperature-sensitive mutant (chemically induced) and a branched-chain amino acid auxotroph (transposon mutant) were also tested as live attenuated vaccines and provided significant protection in mice against challenge with virulent strains.[186,188] Vaccination of mice with an attenuated strain harboring

a suicide plasmid disruption of bipD, a gene encoding a type III secretion system translocation protein, resulted in partial protection against challenge with wild-type *B pseudomallei*.[189] In contrast, vaccination with purified bipD protein did not significantly protect this animal model.[189] More recently, a highly attenuated *B pseudomallei purM* mutant (strain Bp82) was evaluated as a live attenuated vaccine and shown to provide mice significant protection against an intranasal challenge with wild-type *B pseudomallei*.[190] These studies suggest that live attenuated vaccines are promising candidates for melioidosis vaccines; however, strains with defined deletion mutations would be preferred to prevent the possibility of reversion to wild-type.

Iliukhin et al vaccinated guinea pigs with live *B thailandensis* strains and protected less than 50% of the animals challenged with 200 times the LD_{50} of wild-type *B pseudomallei*.[191] *B thailandensis* and *B pseudomallei* produce identical lipopolysaccharide (LPS) O-antigens and contain immunologically related secreted and cell-associated antigens,[112,113,192,193] which probably account for the protection that *B thailandensis* affords. The *B pseudomallei* exopolysaccharide and capsular polysaccharide (CPS; see Table 9-1) are not produced by most *B thailandensis* strains, and both polysaccharides may be necessary for full protection against challenge with *B pseudomallei*. Recently, *B thailandensis* strains (eg, E555) that express the CPS have been identified and tested as vaccine candidates.[194,195] A study by Scott et al showed that immunization with E555 conferred significant protection against a lethal intraperitoneal challenge of *B pseudomallei* in mice.[195] Live attenuated *F tularensis* strains were also tested as heterologous vaccine candidates against melioidosis in rodents.[186,196] Attenuated *F tularensis* strains did afford some protection against challenge with virulent *B pseudomallei*.

A crude acellular melioidosis vaccine was produced to protect captive cetaceans at Ocean Park in Hong Kong.[197] The vaccine consisted of a protein-polysaccharide mixture (1:3), and it significantly protected hamsters against experimental challenge with virulent *B pseudomallei*. The acellular vaccine reduced melioidosis mortality in cetaceans from 45% to less than 1%.[197] Unfortunately, the exact chemical components of the vaccine were not well characterized, leaving a high probability of lot-to-lot variation. A naturally derived outer membrane vesicle vaccine has been developed and tested in BALB/c mice and shown to provide significant protection against a lethal *B pseudomallei* aerosol challenge.[198] In addition, studies have described the testing of purified protein antigens, including LolC (ABC transporter protein), PotF (periplasmic binding protein), OppA (oligopeptide-binding protein), BimA (autotransporter protein), BopA (T3SS effector protein), and Hcp1 (T6SS-1 component) as potential vaccine candidates.[124,199,200] Significant protection was conferred by several of these protein antigens; sterilizing immunity was not.

In a study by Nelson et al, mice were actively immunized with purified *B pseudomallei* CPS or LPS and challenged with virulent *B pseudomallei* by the intraperitoneal or aerosol route.[201] The LPS-vaccinated mice exhibited an increased mean time to death relative to controls, and 50% of the mice survived for 35 days after intraperitoneal challenge. By comparison, mice vaccinated with the purified CPS had an increased mean time to death, but 100% of the vaccinated mice were dead by day 28.[201] Neither of the subunit vaccines provided substantial protection against a lethal aerosol challenge, probably because *B pseudomallei* appears to be more virulent by this route of infection.[102,135] Improved subunit vaccines that generate both humoral and cell-mediated immune responses are probably necessary to protect against infection with *B pseudomallei*.[202]

Several studies have shown that CPS- and LPS-specific monoclonal antibodies (mAbs) can passively protect animals against challenge with *B pseudomallei*.[203–205] The bactericidal and opsonophagocytic activities associated with various anti-LPS or anti-CPS mAbs correlated with their protective capacity in mice.[203] CPS and LPS have also been shown to be major components of an outer membrane vesicle vaccine that provided significant protection in mice.[198] Taken together, such findings indicate that these surface-exposed carbohydrates are protective antigens and support the rationale for developing LPS- and CPS-based vaccines for immunization against melioidosis. Both CPS- and OPS-based glycoconjugates have been produced and shown to be capable of eliciting high-titer, carbohydrate-specific antibody responses; however, the protective capacity of these subunit vaccines remains to be reported.[206,207]

There is no licensed vaccine available to prevent human melioidosis and no definitive evidence that infection with *B pseudomallei* confers immunity, because reinfection with a different strain of *B pseudomallei* has occurred after successful melioidosis treatment.[21] The only proven method of disease prevention for individuals with known risk factors is avoiding *B pseudomallei* in the environment. Recommendations for postexposure prophylaxis (PEP) following a laboratory exposure or bioterrorism event involving *B pseudomallei* are complicated by the lack of efficacy data from either clinical studies or animal experiments. Recommendations are therefore based on expert consensus opinion from physicians who frequently treat melioidosis cases and with data extrapolated from clinical studies on oral

maintenance therapy. The recommended antibiotic for PEP of *B pseudomallei* exposure is TMP-SMX. Dosing is as follows:

- adults greater than 60 kg: 2 × 160 mg/800 mg tablets every 12 hours;
- adults between 40 and 60 kg: 3 × 80 mg/400 mg tablets every 12 hours;
- adults less than 40 kg 2 × 80 mg/400 mg tablets every 12 hours; and
- children: 8 mg/40 mg/kg, maximum dose 320 mg/1,600 mg, every 12 hours.

If the organism is resistant to TMP-SMX or the patient is unable to take sulfa drugs, amoxicillin-clavulanate is the second choice. Dosing is as follows:

- adults greater than 60 kg: 3 × 500 mg/125 mg tablets every 8 hours;
- adults less than 60 kg: 2 × 500 mg/125 mg tablets every 8 hours; and
- children: 20 mg/5 mg/kg every 8 hours, with a maximum dose of 1,000 mg/250 mg every 8 hours.[166,208]

The recommended duration of PEP is 21 days.

SUMMARY

Melioidosis, a disease caused by the saprophytic gram-negative bacterium *B pseudomallei*, is regarded as an emerging infectious disease and a potential bioterrorism threat. *B pseudomallei* is present in water and soil samples in endemic tropical and subtropical regions, and is spread to humans through percutaneous inoculation from a contaminated source or through inhalation or ingestion. The majority of individuals who develop melioidosis have an identifiable risk factor, such as diabetes mellitus, alcoholism, cirrhosis, or other immunosuppressed state, although healthy people may also develop disease. The incubation period is not clearly defined, but may range from 1 to 21 days. Exposed individuals with subclinical infection are potentially at risk for reactivation, which can occur many years later.

Melioidosis has an unusually broad range of clinical presentations. Clinical disease is generally caused by hematogenous seeding of bacteria to various organs within the host, resulting in abscess formation. The majority of patients with melioidosis present to the hospital with bacteremia. Because of its protean clinical manifestations, the diagnosis of melioidosis depends on the isolation and identification of *B pseudomallei* from clinical specimens. Ashdown's selective medium is often used to increase the recovery of *B pseudomallei* from nonsterile clinical specimens. Serologic testing alone is not a reliable method of diagnosis because there is a high prevalence of antibodies to *B pseudomallei* in healthy individuals in endemic areas, and false negative results in patients with sepsis.

All melioidosis cases should be treated with initial intensive therapy followed by oral eradication therapy. *B pseudomallei* is inherently resistant to many antibiotics, which complicates therapeutic decisions. Antibiotics recommended to treat melioidosis are ceftazidime, imipenem/cilastatin, meropenem, and TMP-SMX.

Various experimental melioidosis vaccines have been tested in animal models, but no licensed vaccine exists to prevent human infections. Avoidance of *B pseudomallei* by individuals with known risk factors is the only proven method of disease prevention. The efficacy of PEP in preventing human disease after exposure is unknown, although guidelines based on expert opinion have been published.

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Chapter 10

PLAGUE

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SUMMARY

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INTRODUCTION

Plague, a severe febrile illness caused by the gram-negative bacterium *Yersinia pestis*, is a zoonosis usually transmitted by fleabites. It is foremost a disease of rodents; more than 200 species of mammals have been reported to be infected with *Y pestis*, but maintenance of plague in nature relies almost exclusively on a smaller number of rodent species and their fleas.[1,2] Humans most often become infected by fleabites during an epizootic event; less frequently they are exposed to blood or tissues of infected animals (including ingestion of raw or undercooked meat) or aerosol droplets containing the organism.[1,3] Humans or animals with plague pneumonia, particularly cats, can generate infectious aerosols.[4,5] The resulting primary pneumonic plague is the most severe and frequently fatal form of the disease. Pneumonic plague is of particular concern to the military because it can also be acquired from artificially generated aerosols.

In the 6th, 14th, and 20th centuries *Y pestis* was the cause of three great pandemics of human disease. The bubonic form of the disease in humans is characterized by the abrupt onset of high fever, painful local lymphadenopathy draining the exposure site (ie, a bubo, the inflammatory swelling of one or more lymph nodes, usually in the groin, axillary, or cervical regions; the confluent mass of nodes, if untreated, may suppurate), and bacteremia. Septicemic plague can ensue from untreated bubonic plague or by passage of bacteria directly into the bloodstream bypassing the local lymph nodes. Patients with the bubonic and septicemic forms of the disease may develop secondary pneumonic plague, which can lead to human-to-human spread by the respiratory route. Cervical lymphadenitis has been noted in several human plague cases, including many fatal cases, and is often associated with the septicemic form of the disease. However, it is possible that these patients were exposed by the oral/aerosol route and developed pharyngeal plague that progressed into a systemic infection and cervical lymphadenitis.[1,6–8] According to Dr Kenneth Gage with the Centers for Disease Control and Prevention (CDC) laboratory in Fort Collins, Colorado, in an email in 2006, cervical lymphadenopathy, which is more common in patients from developing countries, may result from fleabites on the neck or face while sleeping on the dirt floors of heavily flea-infested buildings.

During the past 4 millennia, plague has played a role in many military campaigns. During the Vietnam War, plague was endemic among the native population, but US soldiers were relatively unaffected. The protection of troops was largely attributable to the US military's understanding of the rodent reservoirs and flea vectors of disease, the widespread use of a plague vaccine during the war, and prompt treatment of plague victims with effective antibiotics. Mortality from endemic plague continues at low rates throughout the world despite the availability of effective antibiotics. Deaths resulting from plague occur not because the bacilli have become resistant but, most often, because physicians do not include plague in their differential diagnosis, or because treatment is absent or delayed.

To be best prepared to treat plague in soldiers who are affected by endemic disease or a biological agent attack, military healthcare providers must understand the natural mechanisms by which plague spreads between species, the pathophysiology of disease in humans, and the diagnostic information necessary to begin treatment with effective antibiotics. No vaccine is available for plague in the United States, although candidates are undergoing clinical trials.

The US military's concern with plague is both as an endemic disease and as a biological warfare threat. A better understanding of the preventive medicine aspects of the disease will aid in the prompt diagnosis and effective treatment necessary to survive an enemy attack of plague and protect military and civilian personnel in plague endemic areas where military operations are underway.

Key terms in this chapter include enzootic and epizootic. Enzootic refers to when plague is present in a small number of animals; the host, vector, and bacterium live in an apparent equilibrium in which mortality among rodent hosts is difficult to detect and not obviously resulting from plague. During an epizootic, which typically follows a longer period of enzootic maintenance, widespread plague infections frequently lead to death among susceptible host populations (ie, equivalent to an epidemic in a human population), an event that is often noticeable to residents in affected areas. The death of a rodent causes the living fleas to leave that host and seek other mammals, and when those other mammals die in large numbers, they may seek humans. Knowledge of these two concepts of enzootic and epizootic will help to clarify how and when humans may be infected, in endemic or biological warfare scenarios.

HISTORY

The Justinian Plague (The First Pandemic)

Procopius gave us the first identifiable description of epidemic plague in his account of the plague of the Byzantine Empire during the reign of Justinian I (541–542 CE [the common era]), which we now consider to be the first great pandemic of the Common Era.[9] At the height of the epidemic, more than 10,000 people died each day. As many as one hundred million Europeans died, including 40% of Constantinople's population.[10,11] Repeated, smaller epidemics followed this plague into the 8th century.[12] Recently, *Y pestis* was definitively determined to have caused this pandemic based on extraction of plague-specific DNA from the dental pulp of plague victim skeletons in Germany. DNA fingerprinting strongly suggests China as the source of this First Pandemic.[13]

The Black Death (The Second Pandemic)

The second plague pandemic, known as the Black Death, brought the disease into the collective memory of Western civilization.[12] Plague bacilli probably entered Europe via the trans-Asian Silk Road during the early 14th century in fleas on the fur of marmots (a rodent of the genus *Marmota*). When bales of these furs were opened in Astrakhan and Saray, hungry fleas jumped from the fur seeking the first available blood meal, often a human leg.[12,14,15] In 1346, plague arrived in Caffa (modern Feodosiya, Ukraine) on the Black Sea. Caffa's large rat population helped spread the disease as they were carried on ships bound for major European ports such as Pera, a suburb of Constantinople, and Messina, in Sicily. By 1348, plague had already entered Great Britain at Weymouth.[9]

The Black Death probably killed 24 million people between the years 1346 and 1352 and perhaps another 20 million by the end of the 14th century.[10] However, some believe that the plague persisted through 1750, following a final foray into Marseilles in 1720. During the 15th through the 18th centuries, 30% to 60% of the populations of major cities, such as Genoa, Milan, Padua, Lyons, and Venice, died of plague.[15] Debate has raged for decades about the cause of the Black Death. Some believed that a viral hemorrhagic fever or unknown agent caused the Black Death instead of *Y pestis*. *Y pestis* was only recently proven to have caused the Black Death based on finding *Y pestis* DNA and F1 capsular antigen in the dental pulp of plague victims buried in mass graves in England, France, Germany, Italy, and the Netherlands, and later characterization of the genome of a *Y pestis* strain in DNA extracted from the skeletal material in a cemetery where Black Death victims had been buried in England.[16]

Failing to understand the plague's epidemiology and etiology, physicians could offer no effective treatment. Physicians at the University of Paris theorized that a conjunction of the planets Saturn, Mars, and Jupiter at 1:00 PM on March 20, 1345, corrupted the surrounding atmosphere, which led to the plague.[10] Physicians recommended a simple diet; avoidance of excessive sleep, exercise, and emotion; regular enemas; and abstinence from sexual intercourse.[17] Although some people killed cats and dogs because they were thought to carry disease, rats seemed to escape attention.[10] Christians blamed plague on Muslims, Muslims blamed it on Christians, and both Christians and Muslims blamed it on Jews or witches.[12]

In 1666, a church rector in Eyam, Derbyshire, England, persuaded the whole community to quarantine itself when plague erupted, but this was the worst possible solution because the people then remained close to the infected rats and/or fleas. The city experienced virtually a 100% attack rate with 72% mortality. The average mortality for the Black Death was consistently 70% to 80%.[12,18]

Contemporary observers such as Giovanni Boccaccio (1313–1375), who wrote in his *Decameron*, provided accurate clinical descriptions of the Black Death:

> The symptoms were not the same as in the East, where a gush of blood from the nose was a plain sign of inevitable death, but it began both in men and women with certain swellings [buboes] in the groin or under the armpit. They grew to the size of a small apple or an egg, more or less, and were vulgarly called tumours. In a short space of time these tumours spread from the two parts named all over the body. Soon after this, the symptoms changed and black or purple spots appeared on the arms or thighs or any other part of the body, sometimes a few large ones, sometimes many little ones.[19]

Marchione di Coppo Stefano Buonaiuti (1327–1385) wrote in his memoir about the Black Death in Florence:

> In the year of our Lord 1348 there occurred in the city and contado of Florence a great pestilence and such was its fury and violence that in whatever household it took hold, whosoever took care of the sick, all the carers died of the same illness, and almost nobody survived beyond the fourth day, neither doctors nor medicine proving to any avail . . . those symptoms were as follows: either between the thigh and the body, in the groin region, or under the armpit, there

> appeared a lump, and a sudden fever, and when the victim spat, he spat blood mixed with saliva, and none of those who spat blood survived. Such was the terror this caused that seeing it take hold in a household, as soon as it started, nobody remained: everybody abandoned the dwelling in fear, and fled to another; some fled into the city and others into the countryside . . . sons abandoned fathers, husbands wives, wives husbands, one brother the other, one sister the other. The city was reduced to bearing the dead to burial . . ..[20]

Some writers described bizarre neurological disorders (which led to the term "dance of death"), followed by anxiety and terror, resignation, blackening of the skin, and death. The sick emitted a terrible stench: "Their sweat, excrement, spittle, breath, [were] so foetid as to be overpowering" [in addition, their urine was] "turbid, thick, black, or red."[10]

The second great pandemic slowly subsided in Europe by 1750. The pandemic's decline has been attributed to the replacement of the black rat (*Rattus rattus*) in the area by the Norwegian rat (*Rattus norvegicus*), which is a less efficient host; natural vaccination of animals and/or humans by other *Yersinia* species or by less virulent *Y pestis* strains; and other less plausible hypotheses. The theories are all flawed to some extent, and the disappearance of plague from Europe remains one of the great epidemiological mysteries.[3,8,21]

It is not known why plague spread so easily during the First and Second Pandemics and had such a high case-fatality rate. Based on an analysis of *Y pestis* genomes from Black Death victims, no significant differences existed between 14th century and 21st century strains of the bacterium.[22] The environment, vector dynamics, and host susceptibility likely contributed to the disease spread. Although there are legitimate reasons to question the Oriental rat flea's role in parts of northern Europe where it and black rats appeared absent, the human flea's role in transmitting plague to humans in these regions probably has been underemphasized, and could explain the rapid human-to-human spread during the Black Death.[23–25] It also is possible that human lice became vectors during pandemics. Even though lice are not normal vectors, they are capable of transmitting *Y pestis* infection in rabbits, and presumably humans.[26]

The Third Pandemic

The third, or modern, plague pandemic arose in 1894 in China and spread throughout the world as rats and their fleas traveled via modern transportation.[12,18] In 1894, both Shibasaburo Kitasato and Alexandre JE Yersin independently discovered the plague bacillus. However, it was Yersin who was able to satisfy Koch's postulates for the bubonic plague and his plague bacillus fits the characterization of *Y pestis*.[6] The reservoir of plague bacilli in the fleas of the Siberian marmot was likely responsible for the Manchurian pneumonic plague epidemic of 1910 through 1911, which caused 50,000 deaths.[27] The modern pandemic arrived in Bombay in 1898, and during the next 50 years, more than 13 million Indians died of rat-associated plague.[27–29]

The disease officially arrived in the United States in March 1900, when a plague-infected Chinese laborer's lifeless body was discovered in a hotel basement in San Francisco, California. The disease subsequently appeared in New York City and Washington state the same year.[30,31] The disease appeared in New Orleans, Louisiana, in 1924 and 1926.[31] The Texas Gulf Coast and Pensacola, Florida, also saw the influx of plague. Among these numerous events, only the outbreaks in California appear to have spread inland, leading to the establishment of permanent plague foci in native rodent and flea populations in the interior of the western United States.[32,33] Human plague in the United States was initially a result of urban rat and ground squirrel epizootics until 1925. After general rat control and hygiene measures were instituted in various port cities, urban plague vanished—only to spread into rural areas, where virtually all cases in the United States have been acquired since 1925.[34] Rodents throughout the western United States were probably infected from the San Francisco focus.

PLAGUE AND WARFARE

It is an axiom of warfare that battle casualties are much fewer than casualties caused by disease and nonbattle injuries.[35] *Y pestis* can initiate disease both through exposure to natural sources of infection, such as fleabites, and to a biological warfare agent. Medical officers need to distinguish cases likely acquired from natural sources in a plague-endemic region from those occurring following exposure to a biological warfare agent.

Endemic Disease

Plague has also afflicted armies in more recent times. In 1745, Frederick the Great's troops were devastated by plague. Catherine the Great's troops returned from the Balkans with plague in 1769 through 1771. French military operations in Egypt were significantly impeded by plague in 1798, which caused them to abandon their attack on Alexandria. The modern pandemic

began in China when its troops were deployed in an epidemic plague area to suppress a Muslim rebellion. Military traffic is responsible for the rapid plague spread to nearly every country in Asia.[27]

Endemic plague has not been a source of disease and nonbattle injuries for the US military since the mid 20th century. During World War II and the Vietnam War, US military forces were almost free of plague, although civilian populations in certain areas were subjected to plague outbreaks in both of these wars. However, plague remains on and near military bases in the southwestern United States because the local mammal populations are reservoirs of infection, and it may be found in many areas around the world where US military forces are deployed.

World War II

Endemic plague became established in Hawaii (on the islands of Hawaii and Maui) in December 1899. However, no evidence of the disease in either rodents or humans has been found on Oahu or Kauai since the first decade of the 20th century. A "small outbreak" occurred during World War II on the island of Hawaii (in 1943), but it was contained by strict rat control measures, which prevented any plague spread to military personnel during the war in the Pacific.[36] Official policy during World War II was to vaccinate US troops with the whole-cell killed plague vaccine. No troops contracted plague despite serving in known endemic areas.[36,37] Plague has since disappeared from Hawaii.

Vietnam War

Plague entered Vietnam in Nha Trang in 1898 and several pneumonic epidemics have occurred since then.[27,38,39] Cases were reported in Vietnam every year from 1898 to 2002, except during the Japanese occupation during World War II.[27,40] When French forces departed Vietnam after the Indochina War, public health conditions deteriorated and plague flourished. The reported plague incidence increased from eight cases in 1961 to 110 cases in 1963, and to an average of 4,500 cases from 1965 through 1969.[34,41–44] The mortality in clinically diagnosed cases was between 1% and 5%. In untreated individuals, it was much higher (60%–90%).[27,42] However, only eight American troops were affected (one case per 1 million human-years) during the Vietnam War.[44] The low infection rate in the US troops was attributed to insecticide use, vaccination of virtually all troops, and a thorough understanding of plague's epidemiology, which led to the insect repellent use, protective clothing, and rat-proofed dwellings.[27,42] During this period, knowledge of plague grew dramatically, in large part due to the work of two officers of the US Army Medical Service Corps, Lieutenant Colonel Dan C Cavanaugh and Lieutenant Colonel John D Marshall. These scientists studied plague ecology, related plague epidemics to weather, described the effects of high temperatures (>28°C) on the abilities of fleas to transmit plague, developed serologic tests for plague infection, and significantly contributed to the field of plague vaccinology.[27,45]

Disease Threat on US Military Installations

Human exposure to plague on military installations may occur when pets bring home infected rodents or fleas, at recreation areas with sick or dead rodents and their infected fleas, or at field training and bivouac sites. The consequences of plague at a military installation include morbidity and mortality of both humans and pets; loss of training and bivouac sites; large expenditures of money, personnel, and equipment to eliminate the plague risk; and the loss of recreation areas.[34] Plague risk has been identified on and near several US military installations (Exhibit 10-1). For a description of relevant rodent/flea complexes found in the United States, see the Epidemiology section.

Plague as a Biological Warfare Agent

The first known attempt at what is now called "biological warfare" is purported to have occurred at the Crimean port city of Caffa on the Black Sea in 1346 and 1347.[10,27] During the conflict between Christian Genoese sailors and Muslim Tatars, the Tatar army was struck with plague. The Tatar leader catapulted corpses of Tatar plague victims at the Genoese sailors. The Genoese became infected with plague and fled to Italy. However, the disease was most likely spread by the local population of infected rats, not by the corpses, because an infected flea leaves its host as soon as the corpse cools.[10]

The 21st century use of plague as a potential biological warfare weapon is the immediate concern of this chapter. Medical officers need to consider this use of plague, particularly if the disease appears in an unlikely setting.

World War II

During World War II, Japan established a secret biological warfare research unit (Unit 731) in Manchuria, where pneumonic plague epidemics occurred from 1910 through 1911, 1920 through 1921, and 1927; a cholera epidemic also spread in 1919. General Shiro Ishii, the physician leader of Unit 731, was fascinated

EXHIBIT 10-1

PLAGUE RISKS AT US MILITARY INSTALLATIONS*

Plague-infected animals on the installation; human case reported on post:
- Fort Hunter Liggett, California
- US Air Force Academy, Colorado†

Human case reported in the same county:
- Edwards Air Force Base, Colorado‡
- FE Warren Air Force Base, Wyoming
- Kirtland Air Force Base, New Mexico§
- Peterson Air Force Base, Colorado

Plague-infected animals on the installation:
- Dugway Proving Ground, Utah
- Fort Carson, Colorado
- Fort Ord, California
- Fort Wingate Army Depot Activity, New Mexico
- Marine Corps Mountain Warfare Training Center, Bridgeport, California
- Navajo Army Depot Activity, Arizona
- Pueblo Army Depot Activity, Colorado
- Rocky Mountain Arsenal, Colorado
- Vandenberg Air Force Base, California
- White Sands Missile Range, New Mexico

Plague-infected animals or fleas in the same county but not on the installation:
- Bridgeport Naval Facility, California
- Camp Roberts, California
- Dyess Air Force Base, Texas
- Fort Bliss, Texas
- Fort Lewis, Washington
- Sierra Army Depot, California
- Tooele Army Depot, Utah
- Umatilla Army Depot Activity, Oregon
- Nellis Air Force Base, Nevada

No plague-infected animals or fleas on the installation or in the county, but susceptible animals present:
- Fort Huachuca, Arizona

*Does not include military installations near Los Angeles and San Francisco, California, where urban plague cases and deaths were common in the first quarter of the 20th century; no plague cases have occurred in these urban areas since the mid 1920s.
†Fatality: 18-month-old child died of pneumonic plague; rock squirrels and their fleas had taken up residence in the ducts of the child's on-base house.
‡Two human cases in the same county in 1995; animal surveillance on base began in 1996.
§Plague-infected animals in the county in 1995; last human case in the county in 1993; no animal surveillance on base since 1986.
Data sources: (1) Harrison FJ. *Prevention and Control of Plague.* Aurora, CO: US Army Center for Health Promotion and Preventive Medicine, Fitzsimons Army Medical Center; September 1995: 3–8. Technical Guide 103. (2) Data collected from Preventive Medicine Officers on 30 military bases in the United States, March 1996.

by plague because it could create casualties disproportionate to the number of bacteria disseminated, the most dangerous strains could be used to make a lethal weapon, and its origins could be concealed to appear as a natural occurrence. Early experiments, however, demonstrated that aerial bomb dropping of bacteria had little effect because air pressure and high temperatures created by the exploding bombs killed nearly 100% of the bacteria.[46]

One of Ishii's more frightening experiments was his use of the human flea, *Pulex irritans*, as a stratagem to simultaneously protect the bacteria and target humans. This flea is resistant to air drag, naturally targets humans, and can infect a local rat population to prolong an epidemic. Spraying fleas from compressed-air containers was not successful because high-altitude release resulted in too much dispersion and aircraft had to fly low for safety. However, clay bombs solved these technical difficulties and resulted in an 80% survival rate of fleas.[46]

At 5:00 AM on a November morning in 1941, a lone Japanese plane made three low passes over the business center of Changteh, a city in the Hunan province. This area of China was not a plague endemic area. Although no bombs were dropped, a strange mixture of wheat and rice grains, pieces of paper, cotton wadding, and other unidentified particles were observed falling from the plane. Within 2 weeks, individuals in this same area began dying of plague. No individual who contracted plague had recently traveled outside Changteh. Unlike the zoonotic form of the disease that is typically observed, rat mortalities were not noted until months after the human cases. It was also observed that plague usually spreads with rice shipments (because rats infest the grain) along shipping routes, but the nearest plague epizootic was 2,000 km away by land or river. Furthermore, Changteh exported—rather than imported—rice. These unusual circumstances surrounding the plague outbreak suggest that it may have been of deliberate human origin.[46]

In another incident, on October 4, 1940, a Japanese plane dropped rice and wheat grains mixed with fleas over the city of Chuhsien, in Chekiang province. In November, bubonic plague appeared for the first time in the area where the particles had been dropped. Plague caused 21 deaths in 24 days. On October 27, 1940, a Japanese plane was seen releasing similar particles over the city of Ningpo, in Chekiang province. Two days later, bubonic plague occurred for the first time in that city, resulting in 99 deaths in 34 days. No epizootic disease or excessive mortality was found in the rat population.[46]

Since World War II

During the Korean War, allied forces were accused of dropping on North Korea insects that were capable of spreading plague, typhus, malaria, Japanese B encephalitis, and other diseases. However, no evidence exists to support such claims.[47]

In 1999, Dr Ken Alibek (Kanatjan Alibekov), a former Soviet army colonel and scientist, published a book titled *Biohazard* that illuminates the former Soviet Union's extensive biological weapons program.[48] Alibek describes the weaponization of *Y pestis* (including a powdered form) and the development of genetically engineered organisms, one of which was a *Yersinia* strain producing "myelin toxin" that induced both disease and paralysis in animal models. Alibek states that "In the city of Kirov, we maintained a quota of twenty tons of plague in our arsenal every year."[48] Although the accuracy of details presented in the memoir has been debated in some circles, the former Soviet Union had entire institutes devoted to the study of *Y pestis*.

Other state-sponsored or extremist group efforts to obtain *Y pestis* will likely occur. For example, in 1995, a white supremacist and microbiologist fraudulently purchased vials of lyophilized *Y pestis* from the American Type Culture Collection.[49,50] The intended use of these organisms was never determined but it caused alarm and led to legislation requiring that the transfer of disease causing pathogens be reported (CDC Select Agent Program).[49,50]

THE INFECTIOUS AGENT

Taxonomy

Y pestis, the causative agent of plague, is a gram-negative coccobacillus belonging to the family *Enterobacteriaceae*. The genus was named in honor of Alexandre Yersin, the scientist who originally isolated *Y pestis* during a plague outbreak in Hong Kong in 1894; the species name *pestis* is derived from the Latin for plague or pestilence. Previous designations for this species have included *Bacterium pestis*, *Bacillus pestis*, *Pasteurella pestis*, and *Pesticella pestis*.[51] This species is closely related to two other pathogens of the genus *Yersinia*: *Y pseudotuberculosis* and *Y enterocolitica*. The extensive genetic similarity (>90%) between *Y pseudotuberculosis* and *Y pestis* led to a recommendation that *Y pestis* be reclassified as a subspecies of *Y pseudotuberculosis*.[52] This proposal was not well received, primarily because of the fear that this change in nomenclature would increase the potential for laboratory-acquired plague infections. The most recent molecular fingerprinting analysis of *Y pestis* suggests that this pathogen arose from *Y pseudotuberculosis* through microevolution over the past few millennia, during which the enzootic "pestoides" isolates evolved (see Biochemistry on next page). The pestoides strains appear to have split from *Y pseudotuberculosis* more than 10,000 years ago, followed by a binary split approximately 3,500 years later that led to the populations of *Y pestis* more frequently associated with human disease. The isolation of *Y pestis* "pestoides" from both Africa and Asia suggests that *Y pestis* spread globally long before the first documented plague (Justinian) in 784 CE.[53] Recent phylogenetic analyses suggest that among the *Enterobacteriaceae*, *Y pestis* is more closely related to insect and invertebrate-associated genera (such as *Photorhabdus*, *Serratia*, and *Sodalis*) than to vertebrate-associated genera like *Escherichia* and *Salmonella*.[54]

Morphology

The characteristic "safety pin" bipolar staining of this short bacillus (0.5–0.8 μm by 1.0–3.0 μm) is best seen with Wayson's or Giemsa stain (Figure 10-1). Depending on growth conditions, *Y pestis* can exhibit marked pleomorphism with rods, ovoid cells, and short chains present. A gelatinous capsule, known as the F1 antigen, is produced by the vast majority of strains at 37°C. *Y pestis* is nonmotile, unlike the other mammalian pathogens of the genus that produce peritrichous flagella at growth temperatures lower than 30°C.[51,55]

Growth Characteristics

Y pestis can grow at a broad range of temperatures (4°C–40°C) in the laboratory, with an optimal growth temperature of 28°C. Although *Y pestis* grows well on standard laboratory media, such as

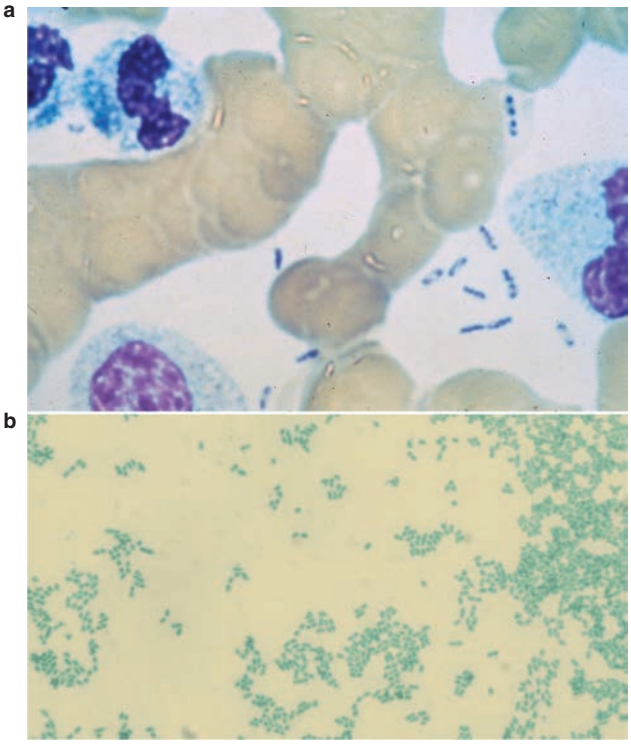


Figure 10-1. (**a**) This Wright-Giemsa stain of a peripheral blood smear from a patient with septicemic plague demonstrates the bipolar, safety-pin staining of *Yersinia pestis*. Gram's and Wayson's stains can also demonstrate this pattern. (**b**) Micrograph of the CO92 strain of *Y pestis* stained with Wayson's stain and examined by microscopy (original magnification × 100).
Photographs: (**a**) Courtesy of Kenneth L Gage, PhD, Centers for Disease Control and Prevention Laboratory, Fort Collins, Colorado. (**b**) Courtesy of Joel Bozue, PhD, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

sheep blood agar, MacConkey agar, or heart infusion agar, growth is slower than that of *Y pseudotuberculosis* or *Y enterocolitica*; more than 24 hours of incubation are required to visualize even pinpoint colonies. Appearance of colonies can be hastened by growth in an environment containing 5% carbon dioxide. The round, moist, translucent, or opaque colonies are nonhemolytic on sheep blood agar and exhibit an irregular edge (Figure 10-2). A fried egg appearance is common in older colonies and is more pronounced in certain strains. Long-term laboratory passage of *Y pestis* or short-term growth under less than optimal conditions is associated with irreversible genetic changes leading to attenuation. These changes include the deletion of a large chromosomal pathogenicity island that encodes factors necessary for growth in both the flea and the mammalian host and the loss of one or more virulence plasmids.[21,51,55] Strains to be archived should be grown at a low temperature and frozen promptly at –70°C.

Biochemistry

Y pestis is a facultative anaerobe, fermenting glucose with the production of acid. It is incapable of a long-term saprophytic existence, partly because of complex nutritional requirements, including a number of amino acids and vitamins. *Y pestis* also lacks certain enzymes of intermediary metabolism that are functional in the closely related but more rapidly growing species such as *Y enterocolitica* or *Y pseudotuberculosis*. *Y pestis* strains have traditionally been separated into three biovars, based on the ability to reduce nitrate (Nit+) and ferment glycerol (Gly+).[21] Some molecular methods of typing, such as ribotyping and restriction fragment-length polymorphisms of insertion sequence locations, support this division of strains.[56,57] Biovar orientalis (Gly^-, Nit^+), which is distributed worldwide and is responsible for the third (modern) plague pandemic, is the only biovar present in North and South America. Biovar antiqua (Gly^+, Nit^+), which is found in Central Asia and Africa, may represent the most ancient of the biovars.[21,53] Biovar mediaevalis (Gly^+/Nit^-) is geographically limited to the region surrounding the

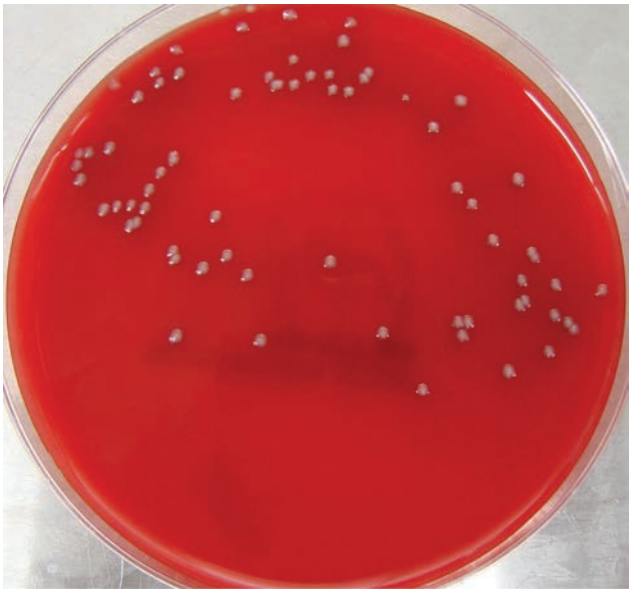


Figure 10-2. Growth of the CO92 strain of *Yersinia pestis* grown on a 5% sheep blood agar plate following 2 days of incubation at 28°C.
Photograph: Courtesy of Joel Bozue, PhD, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

Caspian Sea. No apparent differences in pathogenicity exist among the biovars.[21,58] Recently, three different multilocus molecular methods were used to investigate the microevolution of *Y pestis*.[56] Eight populations were recognized. An evolutionary tree for these populations rooted on *Y pseudotuberculosis* was proposed. The eight population groups do not correspond directly to the biovars; thus, it was suggested that future strain groupings be rooted in molecular typing. Four of the groups were made up of transitional strains of *Y pestis*, "pestoides," which exhibit biochemical characteristics of both *Y pestis* and *Y pseudotuberculosis*.[59] These isolates represent the most ancient of the *Y pestis* strains characterized to date.[53]

EPIDEMIOLOGY

During the modern pandemic, WG Liston, a member of the Indian Plague Commission (1898–1914), associated plague with rats and identified the rat flea as a vector.[27] Subsequently, more than 200 species of mammals and 150 species of fleas have been implicated in maintaining *Y pestis* endemic foci throughout the world, although only a relatively few species play a significant role in disease transmission and maintaining plague in nature.[34,60,61] *Y pestis* is not capable of establishing lasting infections in all flea species, and there appears to be variability in the ability of various flea species to transmit the organism.[58]

The oriental rat flea (*Xenopsylla cheopis*) has been largely responsible for spreading *Y pestis* during bubonic plague epidemics. Some researchers think it is the most efficient flea for transmitting plague.[9] However, this long-held belief has recently been challenged. After the flea ingests a blood meal from a bacteremic animal, bacilli can multiply and eventually block the flea's foregut, or proventriculus, with a biofilm mass containing bacteria as shown in Figure 10-3. When feeding, the flea ingests approximately 0.1 μl to 0.3 μl of blood during a blood meal. High-level bacteremia is a hallmark of *Y pestis* infection in susceptible hosts. This bacteremia provides a sizeable inoculum for the flea and promotes the subsequent infection, and in the case of *X cheopis* it likely leads to blockage of the proventriculus. This blockage limits feeding, resulting in repeated desperate attempts by the flea to feed. Because of the blockage, blood carrying *Y pestis* is regurgitated into the bite wounds, thus spreading the disease to new hosts. The blocked flea, also a victim of the disease, eventually starves to death.[2] As many as 24,000 organisms may be inoculated into the mammalian host.[27] This flea species desiccates rapidly in hot and dry weather when away from its hosts, but flourishes at humidity just above 65% and temperatures between 20°C and 26°C; in these conditions it can survive 6 months without a feeding.[27,34]

Interestingly, the belief that feeding by infectious blocked fleas represents the only means by which *Y pestis* can be transmitted efficiently has recently been challenged as a result of experiments with unblocked *Oropsylla* fleas.[24] These fleas rarely become blocked and were shown to transmit *Y pestis* better. The infected *Oropyslla montana* fleas became infectious within a day after feeding and remained infectious for at least 4 days. This timeframe is much shorter than the 2 weeks required for flea blockage to occur and allows *O montana* and certain other fleas to support the rapid plague spread during epizootics, even in the absence of proventricular blockage.

Although the largest plague outbreaks have been associated with *X cheopis*, all fleas should be considered dangerous in plague-endemic areas.[2,60] During the Black Death, the human flea, *Pulex irritans*, may have aided in human-to-human plague spread; during other epidemics, bedbugs (*Cimex lectularius*), lice, and flies were found to contain *Y pestis*.[9,62] However, the presence of plague bacilli in these latter insects is



Figure 10-3. The oriental rat flea (*Xenopsylla cheopis*) has historically been most responsible for plague spread to humans. This flea has a blocked proventriculus (indicated at the arrow), the equivalent to a human's gastroesophageal region. In nature, this flea would develop a ravenous hunger because of its inability to digest the fibrinoid mass of blood and bacteria. The ensuing biting of the nearest mammal will clear the proventriculus through regurgitation of thousands of bacteria into the bite wound, thereby inoculating the mammal with the plague bacillus.
Photograph: Courtesy of Kenneth L Gage, PhD, Centers for Disease Control and Prevention Laboratory, Fort Collins, Colorado.

associated with ingestion of contaminated blood from plague victims, and they apparently had little or no role as vectors for the disease, although some have recently suggested lice may be important in some situations.[63] In one laboratory study, bedbugs were able to infect guinea pigs after feeding on a moribund *Y pestis*-infected mouse.[64] The most important vector of human plague in the United States is *O montana*, often the most common flea on rock squirrels and California ground squirrels,[34] although cases have been linked to infectious bites of other flea species, including those found on other ground squirrels, prairie dogs, chipmunks, and wood rats.

Throughout history, the black rat, *Rattus rattus*, has been most responsible worldwide for plague's persistence and spread in urban and most rural village epidemics. *R rattus* is a nocturnal, climbing animal that does not burrow, but instead nests overhead and lives close to humans.[9] In the United Kingdom and much of Europe, the brown rat, *R norvegicus*, has replaced *R rattus* as the dominant city rat.[65] Unlike *R rattus*, *R norvegicus* is essentially a burrowing animal that lives under farm buildings and in ditches. Although often considered less important than *R rattus* as a source of *Y pestis* infection, *R norvegicus* may be involved in both rural and urban plague outbreaks.[9] Most carnivores, except wild and domestic cats, are resistant to plague infection, but animals such as domestic dogs, all rodents, and burrowing owls may transport infected fleas into homes. However, in some instances individuals of normally resistant species, such as dogs, can experience serious illness.[66] For example, in 2009 a pneumonic plague outbreak occurred in China. The index case, which was a herdsman who contracted the disease from a sick dog,[67] was unique because it was the first known pneumonic plague case attributed to an infected dog (generally they are considered naturally resistant). Mammals that are partially resistant to plague infection (ie, consist of a mixture of individuals that are either resistant or susceptible to plague-induced mortality) are continuous plague reservoirs. Some epidemiologists propose that the true plague hosts are rodent species with populations consisting of both sensitive and resistant individuals, while others have questioned the need for resistant individuals to maintain plague foci.[68] In the United States, *Cynomous* species (prairie dogs) and *Spermophilus* species (rock squirrels and ground squirrels) are most often associated with plague activity because of the high mortality they often experience during epizootics. A variety of susceptible mammals, such as chipmunks, tree squirrels, cottontail rabbits, ferrets, and domestic cats, are occasionally infected. Epizootic spread among tree squirrels in Denver, Colorado, in the 1960s resulted in the first urban plague case since the 1920s.[60] A more recent epizootic in Denver, Colorado, also involving tree squirrels, occurred in the summer of 2007.[69]

Although not associated with any human plague cases, the appearance of two infected fox squirrels in Dallas, Texas, in 1993 also caused considerable concern.[69,70] An increasing number of human infections has been associated with domestic cats, usually through bites, contact with tissues, suppurating buboes, or aerosol rather than by flea transmission.[4,5] Cats appear to be particularly efficient at transmitting disease to humans.[4,5]

Highly susceptible animals amplify both flea populations and bacilli within their bloodstreams and often support the spread of epizootics, especially when these animals occur at high densities.[71] In many developing countries, these epizootics often involve commensal rat species (*Rattus*) and potential human exposure to infectious rat fleas. In the United States, such epizootics occur in chipmunks, ground squirrels, and wood rats, but especially in prairie dogs, rock squirrels (*Spermophilus variegatus*), and California ground squirrels (*Spermophilus beechyi*). Although prairie dog fleas rarely bite humans, they have been sources of infection for humans, who can acquire the disease by handling infected prairie dogs. Rock squirrels and California ground squirrels both infect humans via direct contact and fleas.[34,72,73] Many other mammals in the United States harbor plague, and a few, including wild carnivores, have served as infection sources for humans (Exhibit 10-2). In 2007, a National Park Service wildlife biologist died of primary pneumonic plague resulting from *Y pestis*, likely contracted from a necropsy

EXHIBIT 10-2

MAMMALS KNOWN TO HARBOR PLAGUE IN THE UNITED STATES

Carnivores	Black bears, cats (including bobcats and mountain lions), coyotes, dogs, foxes, martens, raccoons, skunks, weasels, wolverines, wolves
Rodents	Chipmunks, gophers, marmots, mice, prairie dogs, rats, squirrels, voles
Lagomorphs	Hares, rabbits
Hooved Stock	Pigs, mule deer, pronghorn antelope

Adapted from Harrison FJ. *Prevention and Control of Plague*. Aurora, CO: US Army Center for Health Promotion and Preventive Medicine, Fitzsimons Army Medical Center; September 1995: 25–28. Technical Guide 103.

performed on a dead mountain lion found within Grand Canyon National Park.[74] Thinking that the mountain lion died from trauma, the biologist did not protect himself while removing the lion's skin and skull while performing the necropsy in his garage.

In the United States, where human plague cases are likely to be associated with exposures to native rodents and their fleas rather than rats and rat fleas, knowledge of local host species is critical because certain mammal-flea complexes are particularly dangerous: these complexes consist of both a susceptible mammal genus or species and one or more associated fleas known to bite humans. More than one host-epizootic complex can occur in a given area. These pairings can include the following[34,61]:

- the rock squirrel (*S variegatus*) of the Southwest and the California ground squirrel (*S beechyi*) of California and bordering regions that both are host to the flea *O montana*, which is known to readily bite humans;
- the antelope ground squirrel (*Ammospermophilus leucurus*) and the flea *Thrassus bacchi* of the Southwest;
- the prairie dogs (*Cynomys species*) of the Great Plains, Rocky Mountains, and Colorado plateau region and the flea *Opisochrostis hirsutus*;
- the Wyoming ground squirrel (*Spermophilus elegans*, formerly known as *S richardsoni*) of Colorado and Wyoming or the golden-mantled ground squirrel (*S lateralis*) of the Rocky Mountains and Sierras, and the fleas *Opisochrostis labis*, *Opisochrostis idahoensis*, or *Thrassus bacchi*; and
- various wood rat species (*Neotoma sp*) found across the West and the fleas *Orchopeas sexdentatus* and *Orchopeas neotomae*.

Plague exists in one of two states in nature: enzootic or epizootic. An enzootic cycle is a stable rodent–flea infection cycle presumably occurring in a relatively resistant host population that experiences low mortality. Enzootic maintenance cycles also appear to occur in some areas in more susceptible populations when the animals occur in separate patches or colonies and transmission among them is delayed to some extent by geographical barriers, seasonal changes, or other means.[68,75] In an enzootic cycle, rodent mortality is limited and the fleas have less need to seek alternative hosts, such as humans. During an epizootic, however, plague bacilli also infect moderately or highly susceptible mammals, and infections spread quickly causing high mortality. High mortality occurs, most conspicuously in larger colonial rodents, such as prairie dogs, but it can occur among animals of the relatively resistant rodent populations presumed to be involved in the enzootic cycle, although this tends to be less noticeable.[1] These epizootics are most common when host populations are dense. Evidence has been presented that epizootics and the frequency of human cases are influenced not only by host density but also by climatic variables.[75] Humans are accidental hosts in the plague cycle and are not necessary for the persistence of the organism in nature (Figure 10-4).

Humans typically acquire plague via infectious bites of fleas whose natural host is another mammal, usually a rodent. Infection via flea feces inoculated into skin with bites may also occur, but this mode of transmission is not considered important compared to direct inoculation of the plague bacilli into the feeding site through the flea's mouthparts. Less common infection sources include infectious human fleas, contact with tissues or body fluids from an infected animal, consumption of infected tissues, handling of contaminated pelts, and respiratory droplet transmission from animals with pneumonic disease.[1,3,27,60,61] Fleas removed by humans during the grooming behavior practiced in some cultures are sometimes killed when the person doing the grooming bites the flea, which can squirt the flea's gut contents and viable *Y pestis* into the mouth and pharynx, an act that has been implicated in some cases of plague.[62] The greatest risk to humans occurs when large concentrations of people live under unsanitary conditions in close proximity to large commensal or wild rodent populations that are infested with fleas that bite both humans and rodents.[27]

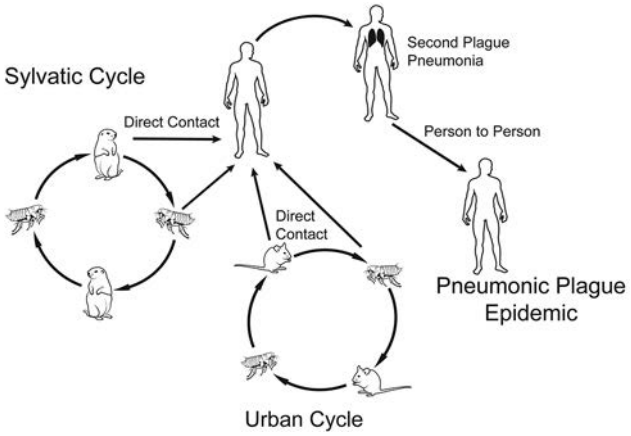


Figure 10-4. This drawing shows the usual, occasional, and rare routes by which plague has spread between various mammals and humans.
Courtesy of William Discher, US Army Medical Research Institute of Infectious Diseases, Visual Information Office, Fort Detrick, Maryland.

Human-to-human plague transmission can occur from patients with pulmonary infection and cough. However, the understanding of pneumonic plague is incomplete. Most large pneumonic epidemics have occurred in cool climates with moderate humidity and close contact between susceptible individuals. Pneumonic plague outbreaks have been rare in tropical climates even during bubonic disease. The role of particle size in efficiency of transmission is unknown, although it may occur more efficiently via larger respiratory droplets or fomites rather than via small-particle aerosols.[76]

Only the pneumonic form of plague can spread between humans. The risk of person-to-person plague transmission via infectious respiratory droplets is lower than once believed. A pneumonic plague outbreak in Madagascar resulting from an index case with secondary pneumonic plague infected 18 individuals and killed 8 of them.[77] However, once the outbreak's cause was determined and appropriate measures were taken, including avoidance of severely ill persons with cough, no person developed infection. Of 154 contacts of these patients who understood the risk, only 8.4% (13/154) developed antibodies to F1 antigen; few were symptomatic and then only had pharyngitis.[77]

Pneumonic plague patients typically transmit disease only several hours before death when they cough up copious amounts of bloody sputum full of bacteria, and then only to individuals who approach them within 1 to 2 meters.[78] The initial pneumonic plague cough is dry. During the Manchurian pneumonic plague epidemics in the first half of the 20th century, prolonged and close contact with end-stage patients were necessary to transmit disease; layered cotton and gauze masks were effective transmission barriers.[78]

A physician with 20 years of experience who cared for 400 to 500 patients with pneumonic plague reportedly has never seen a healthcare worker develop plague from these patients.[78] This record has been attributed to maintaining well-ventilated wards, having patients cough away from healthcare workers during examinations, and limiting time spent close to patients. Most workers in this situation did not have protective masks.

No human-to-human plague transmission cases have been documented after exposure to droplet nuclei (particles <10 microns), which linger for minutes to hours after coughing. All person-to-person transmission seems to be caused by airborne droplets (>10 microns) released immediately during a cough; these droplets rapidly fall to the ground.[77–79] High concentrations of aerosolized droplet nuclei that can transmit plague are used in the laboratory to infect experimental animals.[80] Such small particle aerosols are of particular concern from a biological defense perspective.

INCIDENCE

Under the 2007 revised International Health Regulations, the World Health Organization (WHO) member states are required to report human plague cases in suspected cases in areas not known to be endemic.[81] The cases are then verified, which involves consulting an expert committee to confirm plague based on evidence and additional laboratory testing. Plague may be significantly underreported for several reasons, including the reluctance of some endemic countries to admit to public health problems, difficulties in diagnosis, and the absence of laboratory confirmation. Generally, the distribution of human plague coincides with the geographical distribution of its natural foci.[82,83] In the first decade of the 21st century, 21,725 cases were reported worldwide; 1,612 patients died (7.4%), and 97% of cases were in Africa.[40,84]

Plague is endemic in many countries in Africa, the former Soviet Union, the Americas, and Asia. From recent reports the Democratic Republic of the Congo had 10,581 human plague cases followed by Madagascar (7,182), Zambia (1,309), and Uganda (972). The United States placed 11th with 57 cases, but at least one was reported every year of the decade.[40,82,84]

Since the early 1990s, there have been increasing reports of plague in Africa. It is not clear whether this represents an increase of disease or an improvement of notification to WHO. However, for the Congo, the increase in human plague cases is attributed to civil wars, breakdown in health services, and a greater association of humans with rats.[85–87] Recent plague resurgence in India, Indonesia, and Algeria during the past decade occurred after "silent" periods of years.[82,84] Worldwide distribution of plague and its epidemiology can be found in the *WHO's Plague Manual* (http://www.who.int/csr/resources/publications/plague/). Recent reports of plague activity and occasional summaries of plague activity can be found at the websites for WHO's Weekly Epidemiological Record (http://www.who.int/wer/en/) and the CDC Morbidity and Mortality Weekly Report (http://www.cdc.gov/mmwr/). Known foci of plague are shown in Figure 10-5.

WHO reported 57 deaths among 130 suspected plague cases in the Democratic Republic of the Congo based on a retrospective analysis of cases since December 2004. The victims were employed as miners in a diamond mine at the time of the outbreak. All cases, except

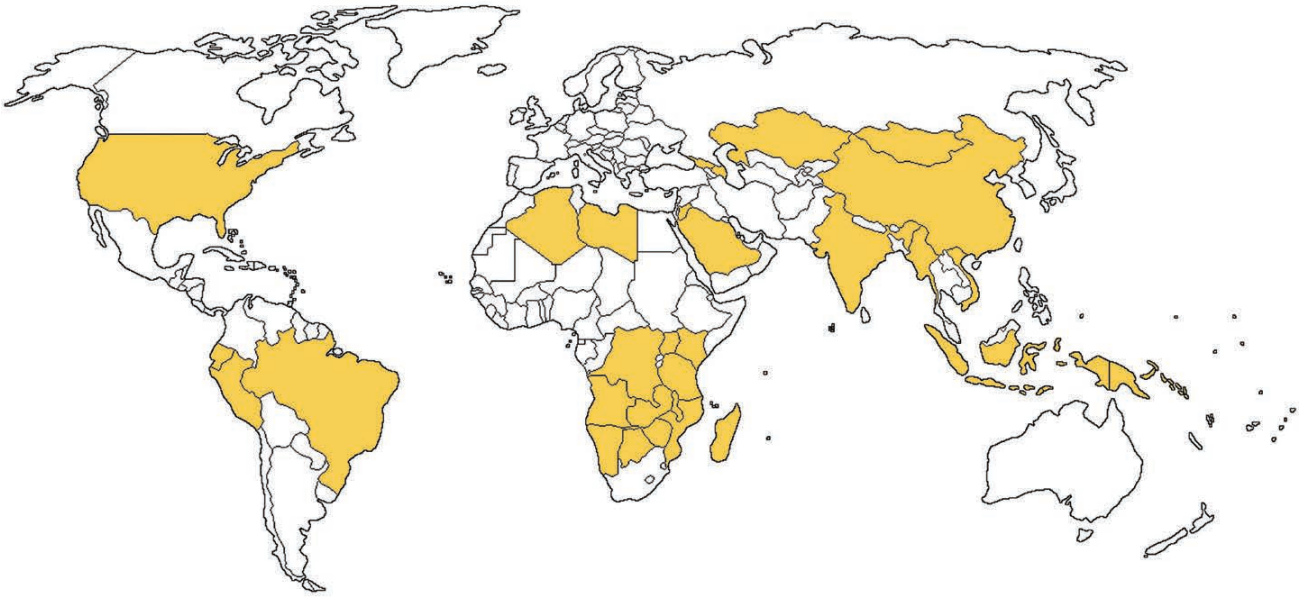


Figure 10-5. Known worldwide foci of human plague infection as indicated by yellow.
Data source: Epidemic Readiness and Interventions, Department of Epidemic and Pandemic Alert and Response, World Health Organization, Geneva, Switzerland.

for two cases of the septicemic form, were reported as pneumonic plague. No evidence of bubonic disease was observed. WHO sent multidisciplinary health teams to investigate the potential outbreak, but no report has been issued since March 2005.[88] The prevalence of pneumonic disease in this group of cases (assuming that this was plague) has not yet been explained.

Plague has been endemic in the continental United States since at least 1900 and now is permanently established from the High Plains on the eastern slope of the Rocky Mountains westward—especially in pine–oak or piñon–juniper woodland habitats at altitudes of 5,000 to 9,000 feet, or on lower, dry grassland or desert scrub areas.[2,30,34,61] In the first quarter of the 20th century, virtually all 432 cases and 284 deaths (65.7% mortality) in the United States occurred in urban port cities.[34] Epidemics occurred in San Francisco, California, from 1900 through 1904 (118 deaths) and from 1907 through 1908 (78 deaths).[34] The last time plague was transmitted between humans in the United States was during the 1924–1925 pneumonic plague epidemic in Los Angeles, California. Eighty percent of cases since 1925 have been sylvatic, involving contact with wild-rodent habitats.[31] Most cases (58%) are in men and occur within a 1-mile radius of home, and half of the US victims have been younger than 20 years old.[31,34]

Between 1926 and 1960, the United States averaged only one plague case per year. This number steadily rose to 3 per year during the 1960s, 11 during the 1970s, and 18 during the 1980s; then it decreased to 9 per year since 1990.[72,73] Between 2000 and 2010, 57 cases were reported; the most active year was 2006 (17 cases).[40,89] The number of states reporting human plague cases has steadily increased over the past 5 decades, most likely because increasing encroachment of humans on previously wild areas brings people closer to infected animals and their fleas.[34] Most human plague cases are reported from New Mexico, Arizona, Colorado, and California.[70,90] In 2002, a couple from New Mexico travelled to New York City and subsequently became ill with plague. However, the couple most likely acquired the infection in an endemic area because *Y pestis* was identified in the dead wood rats and fleas on their property. The bacterial strains recovered from the rats and fleas were indistinguishable from those of the infected couple.[91] In addition, in 2010 two bubonic plague cases from the same household were reported in Oregon, the first in this state since 1995. Most likely, a pet dog's fleas infected the patients. The dog was found to be seropositive.[92]

Epizootic cycles occur approximately every 5 years; the last extremely widespread epizootic with a large die-off of rodents over multiple states (1982–1984) was accompanied by the highest number of humans infected with plague since the urban epidemics of the first quarter of the century.[72,73] In 2009, a fatal laboratory-acquired plague infection occurred by an unknown route with an attenuated

strain of *Y pestis* (KIM D27), which contains defects in the ability to acquire iron.[93,94] However, the scientist was diagnosed portmortem with hemochromatosis. The possibility exists that the excess iron resulting from the condition may have compensated for the iron limitations of the attenuated strain and led to the septicemic infection.[95] This hypothesis was further supported by a recent study that demonstrated virulence can be restored to a *pgm*-strain of *Y pestis* in a mouse model of hereditary hemochromatosis.[95] Before the 2009 case, the last known laboratory-acquired infection in the United States was a pneumonic plague case that occurred in 1959 with a virulent strain of *Y pestis*.[96]

VIRULENCE DETERMINANTS

The persistence of plague in endemic areas requires cyclic transmission between rodents and fleas; thus, *Y pestis* has evolved to survive and replicate in two very different hosts. To maintain the transmission cycle, *Y pestis* must either be transmitted within the few days of the early phase period or multiply within the flea sufficiently to cause blockage and promote the infection of a new mammalian host. Equally critical is the ability to establish an infection and induce a sufficient bacteremia in the mammal to infect fleas during the blood meal. The milieu of the mammalian host is radically different from the flea's midgut, yet, clearly, the organism successfully adapts to each host to complete its life cycle. The adaptation occurs through environmental regulation of virulence factors. For example, gene products necessary for growth in the flea are expressed most efficiently at the flea's body temperature. Likewise, genes required for replication in the mammalian host are expressed at highest levels at 37°C, the internal body temperature of these animals; and the synthesis of some proteins, thought to be induced in the phagolysosome, is also regulated by pH. In the laboratory, the synthesis and secretion of certain essential virulence factors are controlled by both growth temperature and calcium concentration; the induction of these proteins has been termed the low calcium response.[2,21,97,98]

Recent genetic analyses of *Y pestis* and the other pathogenic *Yersiniae* have begun to unravel the unique qualities that make *Y pestis* a successful pathogen in both the flea and the mammalian host. Most strains of *Y pestis* carry three plasmids, two of which are unique to this species:

- pMT (or pFra), which encodes the F1 antigen "capsule"; and
- pPCP, which carries the gene for the virulence factor plasminogen activator.

The third plasmid is common to the human pathogenic *Yersiniae* and is known as pCD (calcium dependence), pYV (*Yersinia* virulence), or pLcr (low calcium response). This plasmid, which is responsible for the synthesis of many antihost factors, is an absolute requirement for virulence.[21]

Type III Secretion System

Like a number of other gram-negative pathogens, the human pathogenic *Yersinae* possess a type III secretion system that enables an organism in close contact to host cells to deliver toxic proteins directly into the eukaryotic cell cytosol.[97,99] In the case of the *Yersinia* species, this system is encoded on the pYV plasmid, which encodes the components of the low calcium response. Toxic activities of the low calcium response effector proteins, designated Yops (***Y****ersinia* **o**uter **p**rotein), include disruption of the cytoskeleton, interference with phagocytic activity, prevention of proinflammatory cytokine synthesis, inhibition of the oxidative burst, and induction of programmed cell death (apoptosis). Yop delivery is necessary for growth of *Y pestis* in the liver and spleen.[100] Specifically, YopM appears to induce a global depletion of natural killer cells. YopH, a protein tyrosine phosphatase, inhibits host cell phagocytosis by dephosphorylating several focal adhesive proteins and inhibiting calcium signaling in neutrophils. YopE, YpkA, and YopT are also antiphagocytic; these toxins inhibit cytoskeletal mobilization. YopJ plays an immunosuppressive role by inhibiting inflammatory cytokine production and inducing apoptosis in macrophages.[97,101,102] Overall, the effect is that of paralyzing professional phagocytes. It is clear why the pathogen-host interaction mediated by the type III secretion system has been designated the "*Yersinia* Deadly Kiss."[102]

LcrV (historically known as V [or "virulence"] antigen), another virulence factor associated with the type III secretion system, is an important protective immunogen in new-generation plague vaccines. This protein serves many roles for the pathogen:

- as regulator of Yop transcription;
- for translocation of Yops into the host cell; and
- as a virulence factor in its own right.[21,101]

LcrV appears to stimulate production of the antiinflammatory cytokine interleukin 10 through interactions with Toll-like receptors 2 and 6 as well as CD14

signaling. These effects appear to be mediated by the N-terminal portion of LcrV.[102,103] Repression of proinflammatory cytokines is presumed to be a result of the interleukin 10 induction. In addition, LcrV released from the cell appears to interact directly with IFN-γ and may contribute to immunosuppression through this binding.[104]

The secretion mechanism includes an "injectisome" that can be visualized as a needle-like structure using electron microscopy. The type III secretion injectisome consists of a cylindrical basal structure spanning the two bacterial membranes and the peptidoglycan, connected to a hollow "needle."[105,106] The needle is tipped by a structure that allows formation of pores in the host cell membrane, and the length of the needle is governed by a protein deemed the "molecular ruler." At body temperature, the secretion apparatus is synthesized on the outer surface of the bacterial cell. Contact with the host cell induces transcription of the Yops and opens this secretion channel that allows the Yops to be translocated through the membrane and into the host cell.[105,106] YopK (also called YopQ) controls the rate of Yop injection from within the host cell.[107] Under certain environmental conditions, proteins with adhesin activity (Ail, Pla, Psa) appear to facilitate Yop delivery.[108]

F1 Antigen

The F1 antigen, encoded by the largest plasmid of *Y pestis* (pMT), is produced in large quantities by *Y pestis* in vivo and when cultured in the laboratory at 37°C. The F1 antigen structure has been described as both capsular- and fimbrial-like because it is composed of fibers that can be shed from the bacteria.[109–112] This capsule-like polymer is generally thought to protect the organism from host phagocytic cells by interfering at the level of receptor interaction in the phagocytosis process.[113] It likely acts in concert with the type III secretion system to provide *Y pestis* with protection from phagocytes. Although the vast majority of natural isolates produce the antigen, F1-negative strains have been isolated from rodent hosts and reportedly from one human case.[110,114–116] In the laboratory, spontaneous mutants defective in F1 production have been obtained from immune animals, cultures treated with antiserum containing F1 antibody, and chronically infected rodents.[114–116] Examination of isogenic F1-positive/-negative strain pairs revealed that F1 is not an absolute requirement for virulence in the mouse and the African green monkey models, including aerosol models, although mutations leading to loss of the capsular antigen increase time to death in the mouse.[114,117] However, a recent study demonstrated a F1 mutant in *Y pestis* was attenuated by bubonic and pneumonic (intranasal) models of infection depending on the strain of mouse.[118] Older studies suggesting a role of F1 in the infection of guinea pigs and rats used F1-negative strains that were not genetically defined and, thus, are more difficult to interpret. However, these studies suggest that the importance of F1 in pathogenesis may vary with the species of the host. The fact that F1-negative strains are relatively rare among natural isolates suggests that the capsular antigen, or other gene products encoded by this plasmid, may play an important role in the maintenance of the disease in animal reservoirs. Historically, F1 has been important as a diagnostic reagent because it is specific to *Y pestis*.[110,119] It is the major antigen recognized in convalescent sera of humans and rodents,[120,121] and also a highly effective protective immunogen.

Other Virulence Factors in the Mammalian Host

Plasminogen Activator

The virulence factor plasminogen activator (Pla) is encoded on a 9.5 kb plasmid, pPCP1, unique to *Y pestis*. Inactivation of the *pla* gene leads to a significant attenuation of virulence from a subcutaneous but not an intraperitoneal or intravenous route of infection in mice, suggesting that Pla promotes dissemination of the organism from peripheral sites of infection, and plasminogen-deficient mice are 100-fold more resistant to *Y pestis* than normal mice.[21,121,122] Although Pla is necessary for full virulence in some *Y pestis* strains, a few strains that are Pla^- and appear to be fully virulent have been identified among natural isolates or generated in the laboratory.[61,122] Presumably, these isolates synthesize other proteins that substitute for Pla function.

Fimbrae

The so-called pH 6 antigen is a fimbrial structure on the surface of *Y pestis* that is necessary for full virulence in the mouse model. Researchers have proposed that pH 6 antigen mediates attachment of the organism to host cells via binding to glycosphingolipids. The temperature and pH of the environment tightly control the biosynthesis of these fimbriae; the expression of pH 6 antigen is most efficient in vitro with a growth temperature between 35°C and 41°C and a pH range of 5.0 to 6.7. This situation suggests that, in vivo, the adhesin activity is likely to be expressed only in specific microenvironments, such as the phagolysosome, necrotic tissue, or an abscess. Intracellular association with macrophages in the laboratory induces synthesis of the fimbriae.[123] More recent data, however, suggest

that the pH 6 antigen does not enhance adhesion to mouse macrophages but rather promotes resistance to phagocytosis.[124] Additional data suggest that this protein is not an essential virulence factor in wild type *Y pestis*; the use of laboratory-passaged strains may have influenced the results of previous studies.[125] Alternatively, there may be redundancy of some functions in *Y pestis* as implied by the work of Felek et al.[108]

Iron and Manganese Sequestration

Acquisition of nutrients in the host is an essential part of pathogenesis. In the mammalian host, iron is sequestered from invading pathogens; therefore, the level of free iron in the extracellular milieu is less than that necessary for bacterial growth. Like most bacterial pathogens, *Y pestis* possesses a high-affinity iron uptake system that is capable of procuring this essential nutrient from the host. Strains that do not produce the low-molecular-weight iron chelator, known as yersiniabactin, or those unable to transport yersiniabactin are highly attenuated by the subcutaneous route of infection and somewhat affected in pneumonic models. Such strains are capable, however, of infecting via the intravenous route (septicemic model). The genes encoding this iron transport system are situated on a chromosomal pathogenicity island known as the pigmentation locus (pgm).[126] Manganese transport is also important for full virulence.[127]

Phage Shock Protein Response

The phage shock protein (PSP) response is almost ubiquitous among microbes; homologues are found in numerous gram-positive and gram-negative bacteria, as well as archaebacteria and even chloroplasts. This regulon appears to respond to environmental stressors, including disturbances in the cell envelope and changes in the proton motive force that are induced by impaired inner membrane integrity.[128,129] For pathogens, environmental stressors triggering the PSP regulon likely include environments within the host, and the PSP response is associated with virulence in *Salmonella enterica*, *Yersinia enterocolitica*, *Yersinia pseudotuberculosis*, and Y *pestis*.[128,130,131] For *Y pestis*, it is required for virulence by both the aerosol and subcutaneous routes of infection.[131]

Twin Arginine Transport

Gram-negative bacteria have numerous ways to transport molecules across their membranes. One of these mechanisms is the twin arginine transport (Tat) pathway. The Tat pathway secretes folded proteins that are identified by an N-terminal signal peptide containing a twin arginine motif across the inner membrane. The TatA gene product mediates the actual translocation event,[132] and it is an important virulence factor of *Y pestis* in both bubonic and pneumonic models of infection.[133] The *tatA* mutation in *Y pestis* leads to many phenotypic changes, including a defect in the secretion/assembly of the F1 antigen on the cell surface. However, the attenuation of a *Y pestis tatA* mutant cannot be explained by the defect in F1 synthesis; the *tatA* mutant is more attenuated than mutants affected in the capsular synthetic genes per se.[133]

Surface Structures

Bacterial surface structures, such as porins and phage receptors, have been implicated in virulence. OmpA, a major outer membrane porin, was identified as an in vivo-expressed protein and subsequently proven to be essential for virulence.[134] Receptors for *Y pestis*-specific bacteriophage also play an important role in virulence; these tend to be associated with various portions of the lipopolysaccharide inner and outer core.[135]

Small RNAs

Posttranscriptional control of virulence determinant expression by small RNAs was recently documented in *Y pestis*.[136] Expression of the majority of small RNAs in *Y pestis* is dependent on the RNA-binding regulatory protein Hfq. Hfq is necessary for growth of the organism at 37°C and for virulence.[136] Identification of regulons governed by small RNAs may lead to identification of virulence factors previously unknown.[137]

Yersinia Autotransporter Proteins

Bacterial autotransporters proteins are secreted via type V secretion pathway and have three conserved domains necessary for mediating secretion. An aminoterminal signal peptide targets the autotransporter to the general secretory pathway for secretion across the inner membrane. From the periplasm, the proteins are translocated to the outer membrane for tethering to the bacterial surface for release following proteolytic cleavage. Numerous autotransporters have been established to be virulence factors in many bacterial pathogens.[138–140] The genome of *Y pestis* encodes for numerous functional *Yersinia* autotransporter proteins (Yaps).[141–143] Many of these Yap genes have increased expression during infection of mammals,[141] and the proteins are necessary for efficient adherence to host cells and colonization of the mammalian host.[142,144,145]

Virulence and Transmission Factors in the Flea

Researchers have identified many factors that allow *Y pestis* to block the flea and promote vectorborne transmission.[146] *Y pestis* has a natural resistance to antimicrobial peptides at growth temperatures similar to that of the flea gut. Such peptides are an integral part of the insect immune system.

Blockage of the proventriculus occurs as a result of bacterial aggregates embedded in a biofilm synthesized by the bacterium at temperatures lower than 28°C. This allows the organism to persist in the proventriculus despite the shearing forces that flush nonaggregating cells into the midgut.[147] The two-component regulatory system, *phoP-phoQ*, contributes to stable biofilm production in the flea.[148] The chromosomal *hmsHFRS* operon, part of the *Yersinia* pathogenicity island ("pgm"), encodes the polysaccharide extracellular matrix that is essential to biofilm formation. The temperature-dependent biofilm synthesis is posttranscriptionally regulated.[149] Although Hms^- mutants are capable of colonizing the flea midgut, they are unable to colonize the proventriculus and, therefore, do not block the flea. The *hms* operon mediates storage of hemin or Congo red in the outer membrane of *Y pestis* on agar medium containing these compounds. This "pigmentation" phenotype, or Pgm, has been associated with virulence of *Y pestis* in animal models; however, Hms per se does not appear to play a role in mammalian plague other than promoting flea transmission. The spontaneous loss of pigmentation in the laboratory usually results from a large chromosomal deletion affecting not only the genes necessary for the Hms phenotype, but also the genetically linked yersiniabactin uptake system. The absence of the high affinity iron transport system in Pgm strains, rather than the loss of Hms, is responsible for attenuation in animal models.[126]

Studies examining the role of the *Y pestis* plasmids in the flea host indicated that one or more genes on the plasmid pMT are necessary for colonizing the midgut.[150] The so-called murine toxin encoded by this plasmid appears to be one of these colonization factors. Murine toxin has phospholipase D activity, and although toxic to mice and rats in pure form, it is not important for virulence in rodent models.[150] This may be explained by the regulation of toxin synthesis. Like Hms, it is produced more efficiently at 28°C than at mammalian body temperatures. Ymt, the *Yersinia* murine toxin, appears to protect the bacterium from an unidentified antibacterial substance in the midgut.[146]

PATHOGENESIS

As few as 1 to 10 *Y pestis* organisms are sufficient to cause infection by the oral, intradermal, subcutaneous, or intravenous routes.[21] Estimates of infectivity by the respiratory route for nonhuman primates vary from 100 to 20,000 organisms.[110,151,152] After being introduced into the mammalian host by a flea, where it had been at ambient temperature, the organism is thought to be initially susceptible to phagocytosis and killing by neutrophils. In rodent models of bubonic plague, it has been shown that neutrophils are quickly recruited to the area associated with the bacteria. Furthermore, the ability to evade and neutralize neutrophils was necessary for infection.[153] However, some of the bacteria may grow and proliferate within tissue macrophages.[154] A vigorous debate has raged for many years regarding the relative importance of intracellular versus extracellular replication in plague. Although most of the bacterial multiplication in the mammalian host is extracellular, evidence indicates that *Y pestis* can survive and multiply in macrophages. As reviewed by Pujol and Bliska, growth inside host cells is likely to be of greatest importance at the early stages of colonization.[155] They suggest that, although considerable attention has focused on how *Y pestis* subverts the functions of phagocytes from the outside, there is less understanding of how these bacteria affect macrophage functions from the inside.[155] Once the antiphagocytic gene products are expressed, the bacteria are resistant to phagocytosis and multiply extracellularly. However, another recent study demonstrated that a *Y pestis* mutant strain severely defective for intracellular recovery within macrophages was still fully virulent in a murine pneumonic plague challenge.[156] The ability of *Y pestis* to reside and replicate in an intracellular environment may be dependent upon the route of infection (parenteral versus respiratory).

During the incubation phase, the bacilli most commonly spread to regional lymph nodes, where lymphadenitis develops, producing the characteristic bubo. Dissemination from this local site leads to septicemia and seeding of other organs, including the liver, spleen, lung, and (less often) the meninges. The endotoxin of *Y pestis* probably contributes to the development of septic shock, which is similar to the shock state seen in gram-negative sepsis from other causes. The endotoxin may also contribute to the resistance of the organism to the bactericidal activity of serum.[151]

Primary pneumonic plague, the most severe form of disease, arises from inhalation of infectious respiratory droplets or an aerosol. Primary pneumonic plague is more rapidly fatal than secondary.[1] During primary

pneumonic plague, the disease can be divided into two host response phases. During the initial preinflammatory phase, the bacteria are actively replicating in the absence of host innate immune responses. Next, a proinflammatory phase occurs with a neutrophil influx, proinflammatory cytokine storm, and tissue destruction within the lung. Evidence suggests that *Y pestis* suppresses the host immune cells in the lung early during infection. As discussed above, the type III secretion system Yop effectors act upon numerous mammalian cells to elicit antiinflammatory and antiphagocytic effects. During pneumonic infection, the type III secretion system initially targets macrophages and neutrophils.[157]

Primary septicemic plague can occur from direct inoculation of bacilli into the bloodstream, bypassing initial multiplication in the lymph nodes. Asymptomatic pharyngeal carriage of plague has occurred in contacts of patients with either bubonic or pneumonic plague.[77,158,159]

CLINICAL MANIFESTATIONS

From 1947 through 1996, 390 cases of plague were reported in the United States, resulting in 60 (15.4%) deaths.[70,91] Of these deaths, bubonic plague accounted for 327 cases (83.9%) and 44 deaths (13.5%); primary septicemic plague accounted for 49 cases (12.6%) and 11 deaths (22.4%); and primary pneumonic plague accounted for 7 cases (1.8%) and 4 deaths (57.1%).[70,91] Seven cases (1.8%) were unclassified, including 1 death (14.3%).[70,160] If *Y pestis* was used as a biological warfare agent, the clinical manifestations of plague would be (*a*) rapidly progressive, highly fatal epidemic pneumonia if aerosolized bacteria were used, or (*b*) bubonic and septicemic plague if fleas were used as carriers. Infections via ingestion could also occur.[1]

Bubonic Plague

Human symptoms of bubonic plague typically develop 2 to 8 days after being bitten by an infected flea. Presenting symptoms include prostration or severe malaise (75% of cases), headache (20%–85% of cases), vomiting (25%–49% of cases), chills (40% of cases), altered mentation (26%–38% of cases), cough (25% of cases), abdominal pain (18% of cases), and chest pain (13% of cases).[27] In the United States, fleabites typically occur in the lower extremities; therefore, buboes are most common in the femoral and inguinal regions. As noted previously, the proportion of bubonic cases with cervical buboes is often higher in poverty-stricken areas of developing countries because these cases involve persons that frequently sleep on the dirt floors of huts where fleas are likely to bite them on the head and neck. Infection arising from skinning plague-infected animals typically produces axillary buboes due to inoculation of the upper extremities. Six to 8 hours after onset of symptoms, buboes, heralded by severe pain, appear (Figure 10-6). Buboes may drain spontaneously and rarely require incision and drainage because of pronounced necrosis. Within 2 days, a warm, erythematous bubo can grow to the size of an egg and eventually reach 10 cm in diameter as a cluster of infected lymph nodes. Buboes are so intensely painful that even nearly comatose patients attempt to shield them from trauma and will abduct their extremities to decrease pressure. Buboes are often associated with considerable surrounding edema, but lymphangitis is rare. Occasionally, buboes become fluctuant and suppurate. Histologically buboes demonstrate hemorrhagic necrosis, numerous neutrophils, a plethora of extracellular bacteria, and completely destroyed lymph node architecture.[40]

A small minority of patients bitten by plague-infected fleas develop *Y pestis* septicemia without a discernable bubo. Other manifestations of bubonic plague include bladder distention, apathy, confusion, fright, anxiety, oliguria, and anuria. Tachycardia, hypotension, leukocytosis, thrombocytopenia, and fever are frequently encountered. In about half of untreated cases of bubonic plague, septicemia ensues 2 to 6 days later, which is virtually 100% fatal if untreated.[40,161] In the United States, approximately 10% to 15% of bubonic plague patients have developed secondary pneumonic plague with the potential for airborne transmission of the organism.[162]

Septicemic Plague

Septicemic plague may occur primarily—if the bacteria is inoculated by a fleabite or other means, such as a puncture wound caused by a knife used while skinning an animal or bypass regional lymph nodes—or secondarily as a complication of hematogenous dissemination of bubonic plague.[40] Presenting signs and symptoms of primary septicemic plague are essentially the same as those for any gram-negative septicemia: fever, chills, nausea, vomiting, and diarrhea. Purpura (Figure 10-7), disseminated intravascular coagulation, and acral cyanosis and necrosis (Figure 10-8) may be seen later. In New Mexico between 1980 and 1984, plague was suspected in 69% of patients who had bubonic plague, but in only 17% of patients who had the septicemic form. The mortality was 33.3% for septicemic plague versus 11.5% for bubonic, which indicates the difficulty of diagnosing septicemic plague.[162] Diagnosis of septicemic plague took longer

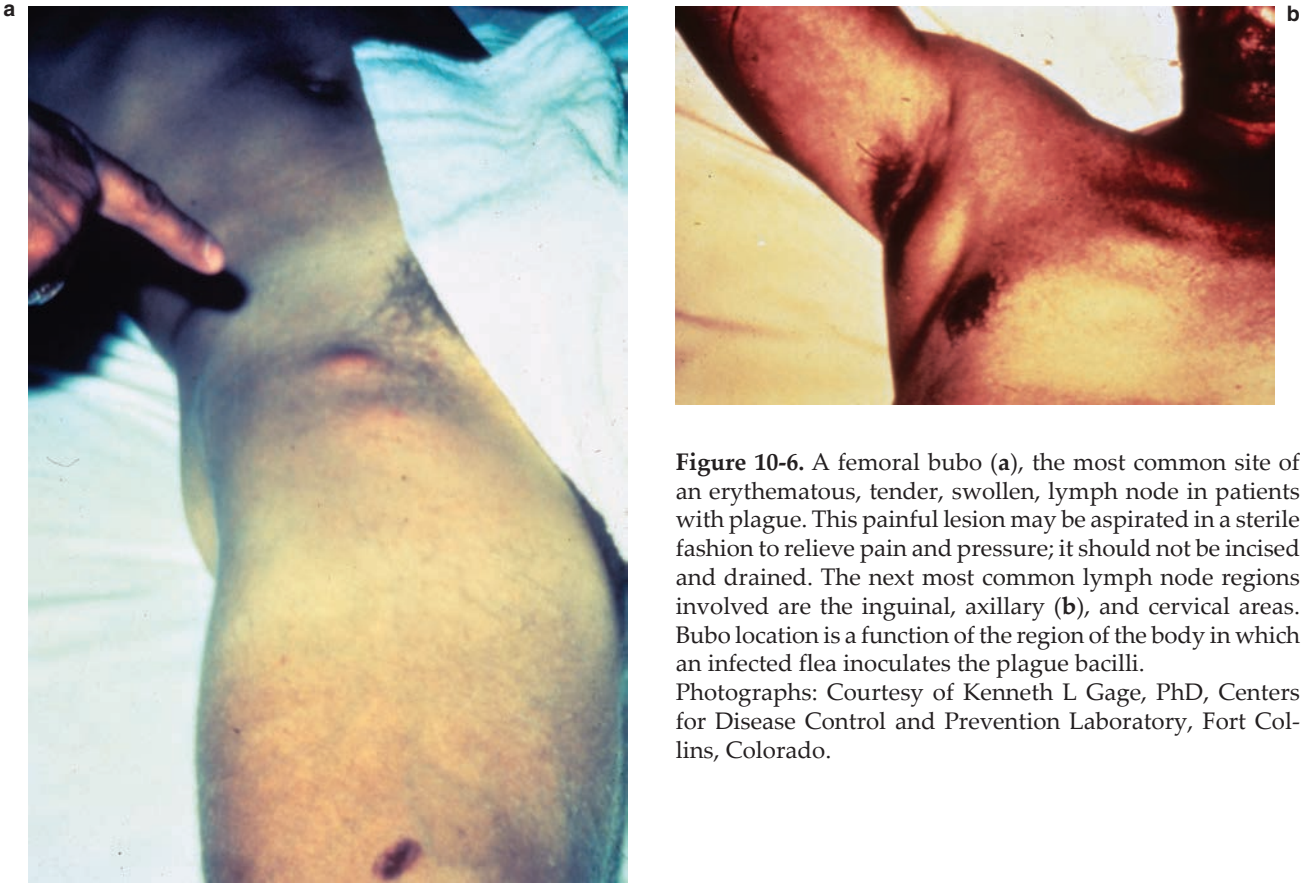


Figure 10-6. A femoral bubo (**a**), the most common site of an erythematous, tender, swollen, lymph node in patients with plague. This painful lesion may be aspirated in a sterile fashion to relieve pain and pressure; it should not be incised and drained. The next most common lymph node regions involved are the inguinal, axillary (**b**), and cervical areas. Bubo location is a function of the region of the body in which an infected flea inoculates the plague bacilli.
Photographs: Courtesy of Kenneth L Gage, PhD, Centers for Disease Control and Prevention Laboratory, Fort Collins, Colorado.



Figure 10-7. Purpuric lesions can be seen on the upper chest of this girl with plague. The bandage on her neck indicates that a bubo has been aspirated.
Photograph: Courtesy of Kenneth L Gage, PhD, Centers for Disease Control and Prevention Laboratory, Fort Collins, Colorado.



Figure 10-8. This patient is recovering from bubonic plague that disseminated to the blood (septicemic form) and the lungs (pneumonic form). Note the dressing over the tracheostomy site. At one point, the patient's entire body was purpuric. Note the acral necrosis of (**a**) the patient's nose and fingers and (**b**) the toes.
Photographs: Courtesy of Kenneth L Gage, PhD, Centers for Disease Control and Prevention Laboratory, Fort Collins, Colorado.

(5 vs 4 days) after onset, although patients sought care earlier (1.7 vs 2.1 days) and were hospitalized sooner (5.3 vs 6.0 days) than patients with bubonic plague. The only symptom present significantly more frequently in septicemic than in bubonic plague was abdominal pain (40% vs <10%), which was probably caused by hepatosplenomegaly.[162]

Pneumonic Plague

Pneumonic plague may occur primarily, from inhaling infectious respiratory droplets or aerosols, or secondarily, from hematogenous dissemination. It is the only form of plague that can be transmitted from one person to another.[78] Patients with pneumonic plague rapidly develop symptoms of a severe bronchopneumonia, severe headache, chills, malaise, tachypnea, tachycardia, dyspnea, cough, chest pain, and hemoptysis.[78,163,164] Patients initially exhibit a dry cough that progressively becomes productive as sputum concentration of blood and bacilli increases to "almost pure culture" levels at the end.[78] The findings on a chest x-ray may be variable, but bilateral alveolar infiltrates appear to be the most common finding (Figure 10-9).[78,165,166] A chest x-ray with bilateral alveolar infiltrates in a bubonic plague patient sometimes represents adult respiratory distress syndrome and disseminated intravascular coagulation in the absence of plague pneumonia.[164] Depending on the stage of infection, the sputum may be clear, purulent, or hemorrhagic, and contain gram-negative rods. Unless appropriate antimicrobial therapy is begun during the first day of symptoms, pneumonic plague is rapidly fatal.[5,40] The time from respiratory exposure to death in humans is reported to have been between 2 to 6 days (and from symptoms to death 1–3 days) in epidemics during the preantibiotic era.[78,163,167]



Figure 10-9. This chest roentgenogram shows right middle- and lower-lobe involvement in a patient with pneumonic plague.
Photograph: Courtesy of Kenneth L Gage, PhD, Centers for Disease Control and Prevention Laboratory, Fort Collins, Colorado.

Plague Meningitis

Plague meningitis is seen in 6% to 7% of cases. The condition manifests itself most often in children after 9 to 14 days of ineffective treatment. Symptoms are similar to those of other forms of acute bacterial meningitis.[165]

Pharyngeal and Gastrointestinal Plague

In late December 2007, the first known cases of plague in Afghanistan developed among 83 persons who developed acute gastroenteritis after eating the meat of a slaughtered, sick camel, and 17 of those patients died.[168]

Transient asymptomatic pharyngeal carriage may occur in healthy contacts of bubonic plague patients.[77,158,159,169] Symptomatic pharyngeal plague presents with pharyngitis, fever, and cervical lymphadenopathy after inhalation of plague bacteria or ingestion of meat from infected camels or goats.[1,3,6,161] For example, in early 1997, 12 individuals in Jordan ate raw or cooked meat from the same (infected) camel and all developed pharyngeal plague. One developed pneumonic plague. However, all survived because they were serendipitously treated with gentamicin for the suspected diagnosis of tularemia.[170]

A plague syndrome of cervical buboes, peritonsillar abscesses, and fulminant pneumonia has been reported in Vietnam[161] and among Indians of Ecuador, who are known to catch and kill fleas and lice with their teeth.[62] Endobronchial aspiration from peritonsillar abscesses may lead to fulminant pneumonia.



Figure 10-10. This child has left axillary bubonic plague. The erythematous, eroded, crusting, necrotic ulcer on the child's left upper quadrant is located at the presumed primary inoculation site.
Photograph: Courtesy of Kenneth L Gage, PhD, Centers for Disease Control and Prevention Laboratory, Fort Collins, Colorado.

Cutaneous Manifestations

While most plague patients have normal appearing skin (apart from buboes), approximately 4% to 10% of patients develop an inoculation-site pustule, ulcer, eschar, or carbuncle (Figure 10-10).[40,154,165,171–173] A sample from a plague patient eschar (ecthyma gangrenosum lesion) grew *Y pestis*, which suggests that local skin lesions are the result of septicemic seeding of the organism.[173]

Petechiae and ecchymoses may develop during hematogenous spread of bacteria when patients develop disseminated intravascular coagulation secondary to the *Y pestis* endotoxin. When purpura and acral gangrene occur, possibly exacerbated by the tissue plasminogen activator, the prognosis is

poor.[27,40,173] Recently, an American man contracted bubonic plague that progressed to septic shock; he developed ischemic necrosis of his feet requiring bilateral foot amputation.[174] Patients in the terminal stages of pneumonic and septicemic plague often develop large ecchymoses on their backs. Lesions like these are likely to have led to the medieval epithet "the Black Death."

DIAGNOSIS

Signs and Symptoms

The early diagnosis of plague requires a high index of suspicion. Presence of a painful bubo in the setting of fever, prostration, and possible exposure to rodents or fleas in an endemic area should readily suggest the diagnosis of bubonic plague. However, if the health-care provider is not familiar with the disease or does not ask the patient for a travel or exposure history, or if the patient presents in a nonendemic area or without a bubo, then the diagnosis will be difficult to make. For example, in the United States in 1996, fatal plague cases occurred in two young adults who presented for treatment without obvious buboes.[175] The first, an 18-year-old male, presented with left groin swelling and tenderness that was misdiagnosed as a groin muscle strain attributed to a fall 2 days earlier. The second, a 16-year-old female, presented with left arm numbness and left axillary pain that was misdiagnosed as a possible brachial plexus injury related to a fall from a trampoline 3 days earlier. In both cases, the patients were sent home with a pain reliever, and they both experienced rapid progression of their illness within the next day and died.[90]

The wildlife biologist who died of pneumonic plague after necropsy of an infected mountain lion did present to a health clinic with fever, chills, nausea, myalgias, and a cough producing blood-tinged sputum.[74] No chest x-ray was performed. No exposure history to wildlife during his job was elicited.[74] Either the chest x-ray or a job exposure history could have saved his life.

The laboratory plague researcher who died in Chicago of septicemic plague presented to an outpatient clinic 3 days before death.[94] He complained of fever, body aches, and a 3-day history of nonproductive cough. Influenza or other acute respiratory infection was suspected, and he was referred to an emergency department, but the patient did not follow through. Neither at that clinic visit nor at his hospital admission 12 hours before death was his occupation noted.[94]

When a bubo is present, the differential diagnosis should include tularemia, cat scratch disease, lymphogranuloma venereum, chancroid, tuberculosis, streptococcal adenitis, and scrub typhus (Figure 10-11). In both tularemia and cat scratch disease, the inoculation site is typically more evident and the patient will usually not be septic. In chancroid and scrofula, the patient has less local pain, the course is more indolent, and there is no sepsis. Patients with chancroid and lymphogranuloma venereum will have a recent history of sexual contact and genital lesions. Those with the latter disease may be as sick as patients with plague. Streptococcal adenitis may be difficult to distinguish initially, but the patient is usually not septic, and the node is more tender when plague is present.

The implications of the absence of a bubo were demonstrated in a review of 27 plague cases seen in New Mexico.[166] In this study, there were eight cases of septicemic plague and 19 cases of bubonic plague, with six fatalities. Of the patients who died, three had septicemic plague and three had bubonic plague, but all six presented with nonspecific febrile symptoms or symptoms of an upper respiratory tract infection. The authors concluded that the lack of a bubo was associated with a delay in the diagnosis of plague and increased mortality.[166]

The differential diagnosis of septicemic plague also includes meningococcemia, gram-negative sepsis, and the rickettsioses. The patient with pneumonic plague who presents with systemic toxicity, a productive cough, and bloody sputum suggests a large differential diagnosis. However, demonstration of gram-negative rods in the sputum should readily suggest the correct diagnosis, because *Y pestis* is perhaps the only gram-negative bacterium that can cause extensive, fulminant pneumonia with bloody sputum in an otherwise healthy, immunocompetent host.

Laboratory Confirmation

Procedures for the isolation and presumptive identification of *Y pestis* by Level A laboratories can be downloaded from the CDC website (http://www.bt.cdc.gov/agent/plague/index.asp).[172] The World Health Organization offers its Plague Manual online (http://www.who.int/emc-documents/plague/whocdscsredc992c.html). A recent review of the methodology for isolating and identifying *Y pestis* from clinical samples and animals is available.[55] Standard bacterial methodologies include staining and microscopic analysis of the organism, isolation on culture medium, and biochemical tests. Misidentification of *Y pestis* by automated systems used for bacterial identification resulting in the delayed diagnosis of human plague has been reported.[175] Therefore, if plague is suspected, immediate appropriate treatment should be started

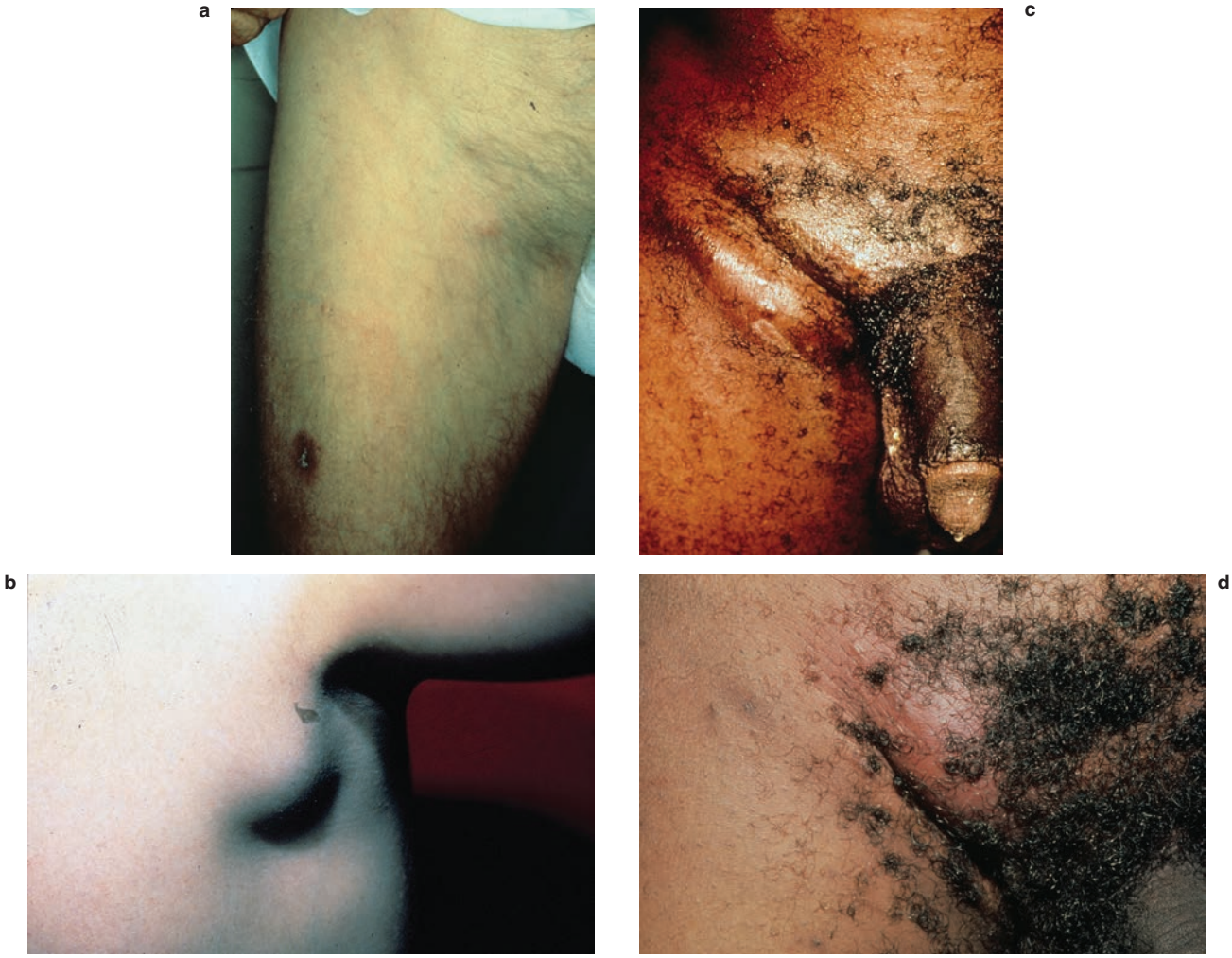


Figure 10-11. (**a**) Small femoral bubo and presumed inoculation site (on the inferior thigh) in a patient with tularemia. This gram-negative bacterial infection (with *Francisella tularensis*) may closely mimic bubonic plague and is successfully treated with the same antibiotics. (**b**) Axillary bubo seen in child with cat scratch disease. (**c**) Greenblatt's sign of ipsilateral femoral and inguinal buboes with intervening depression over the inguinal ligament seen in a patient with lymphogranuloma venereum caused by *Chlamydia trachomatis*. (**d**) Large inguinal bubo seen in a patient with chancroid caused by *Haemophilus ducreyi*. Photographs: Courtesy of Dermatology Service, Fitzsimons Army Medical Center, Aurora, Colorado.

and isolates should be sent to the appropriate laboratories experienced in the identification of *Y pestis*. Care should be taken to avoid aerosols; in this regard, fixing slides with methanol rather than heat fixing is preferred. CDC summarizes diagnosis of plague at its website (http://www.cdc.gov/plague/healthcare/clinicians.html).

Reference laboratories, such as those found in major county or state health departments, have additional tests to confirm the diagnosis of *Y pestis*. These tests include direct fluorescent antibody tests to detect the F1 capsular antigen and polymerase chain reaction based assays, which can be used on isolates or direct clinical samples. Confirmatory testing includes lysis by a species-specific bacteriophage.[1] Serological testing such as passive hemagglutination antibody detection in acute or convalescent-phase plasma or enzyme-linked immunosorbent assay are found at national laboratories such as the CDC at Fort Collins, Colorado, and the US Army Medical Research Institute of Infectious Diseases at Fort Detrick, Maryland.[176] Serological assays measuring the immune response to plague infection are mainly of value, retrospectively, because patients present clinically before they develop a significant antibody response. A four-fold change in antibody between acute and convalescent serum samples is considered confirmatory.

When using the fluorescent antibody test to detect the plague-specific capsular antigen, it is important to recall that F1 antigen is produced only at temperatures

greater than 33°C. Thus, this method requires a relatively fresh sample from the patient/animal or from a laboratory culture incubated at the appropriate temperature. Therefore, flea samples, as well as samples refrigerated for more than 30 hours, are F1 antigen negative.[55] For diagnosing plague in the field, a new rapid diagnostic test with monoclonal antibodies to the F1 antigen has been developed and field tested in Madagascar. The rapid diagnostic test detected concentrations of F1 antigen as low as 0.5 ng/mL in as little as 15 minutes and had a shelf life of 21 days at 60°C. This test had 100% sensitivity and specificity against laboratory isolates of *Y pestis,* and the agreement between field testing and reference laboratory testing was 89.9%. This test demonstrated positive and negative predictive values of 90.6% and 86.7%, respectively.[176] A rapid and reliable test such as the rapid diagnostic test, which healthcare workers can easily perform at the patient's bedside, holds considerable promise for rapid plague diagnosis in endemic countries, but further testing is needed. A polymerase chain reaction test using primers for the plasminogen activator gene (*pla*) can detect as few as 10 *Y pestis* organisms, even from flea tissue. This test may be useful in surveillance of rats and can be adapted to help diagnose human infection.[177] More recently, the use of Pla primers for simulated detection of *Y pestis* in sputum was reported to have a sensitivity of 10^4 CFU/mL and a 5-hour turnaround.[178] In cases where use of *Y pestis* as a biological weapon is a possibility, it should be kept in mind that F1 or Pla are not necessary for virulence in animal models.[59,110,179] Strains lacking these important diagnostic targets may still be threats.

Cultures of blood, bubo aspirate, sputum, and bronchial/tracheal washings, and/or cerebrospinal fluid (if indicated) should be performed based on the clinical presentation. Tiny 1- to 3-mm "beaten-copper" colonies will appear on blood agar by 48 hours, but *Y pestis* is slow growing and cultures may appear negative at 24 hours. In one study, 24 of 25 blood cultures (96%) of patients with bubonic plague were positive on standard supplemented peptone broth.[6] In patients with lymphadenopathy, a bubo aspirate should be obtained by inserting a 20-gauge needle attached to a 10-mL syringe containing 1 mL of sterile saline. Saline is injected and withdrawn several times until it is tinged with blood. Repeated, sterile bubo aspiration may also be done to decompress buboes and relieve pain. Drops of the aspirate should be air-dried on a slide and methanol-fixed for staining. When evaluating stained material, it should be considered that the characteristic bipolar staining is not specific for *Y pestis* nor is it always observed. If available, a direct fluorescent antibody stain of bubo aspirate for the presence of *Y pestis* capsular antigen should be performed; a positive direct fluorescent antibody result is more specific for *Y pestis* than are the other listed stains (Figure 10-12).[180]

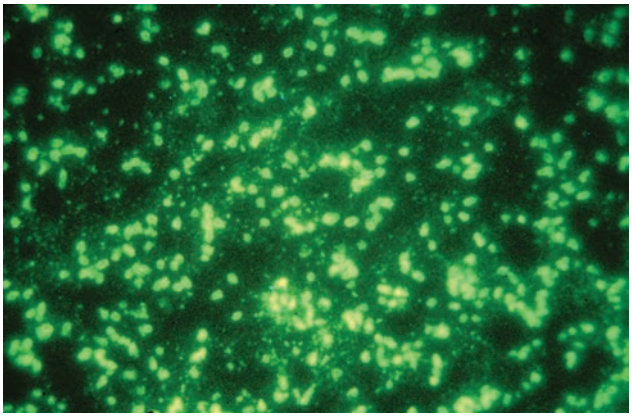


Figure 10-12. These *Yersinia pestis* fluorescent cells are from an infected mouse spleen. Notice how the outlines of the coccobacilli "light up" in this direct fluorescent antibody (DFA) test. The DFA test is specific and therefore better than the other stains discussed in this chapter (original magnification × 1,000).
Photograph: Courtesy of MC Chu, Centers for Disease Control and Prevention Laboratory, Fort Collins, Colorado.

In patients with plague, complete blood counts often reveal leukocytosis with a left shift. Leukemoid reactions with up to 100,000 white blood cells/μL may be seen, especially in children. Platelet counts may be normal or low, and partial thromboplastin times are often increased. Leukocytosis with thrombocytopenia is common in plague, but rare in other conditions.[181] When disseminated intravascular coagulation is present, fibrin degradation products will be elevated. Because of liver involvement, alanine aminotransferase, aspartate aminotransferase, and bilirubin levels are often increased.

TREATMENT

Isolation

Previous editions of this chapter recommended strict isolation until patients had received treatment for 48 hours. However, only standard infection precautions are necessary when caring for bubonic and septicemic plague patients, whereas droplet precautions are still recommended until pneumonic plague patients have received 48 hours of antibiotic therapy. However, such droplet precautions are strictly only necessary when patients are coughing up of bloody sputum. Simply wearing masks, practicing good

hand hygiene, and avoiding close contact (within 2 m) will almost always prevent pneumonic plague transmission.[77,182]

Microbiology laboratory personnel must be alerted when *Y pestis* is suspected because laboratory-acquired plague cases have been reported in the United States.[92,96] Treatment of plague is summarized on the CDC website (http://www.cdc.gov/plague/healthcare/clinicians.html).

Antibiotics

Both because of difficulty acquiring streptomycin and its unfavorable side effect profile, the Working Group on Civilian Biodefense and the CDC now recommend gentamicin as a first-line alternative to streptomycin.[79,183] Gentamicin is given 5 mg/kg intramuscularly (IM) or intravenously (IV) once daily, or 2 mg/kg loading dose followed by 1.7 mg/kg IM or IV three times daily.[79] A recent review of 75 cases of human plague in New Mexico demonstrated that gentamicin alone or in combination with a tetracycline was as efficacious as streptomycin for treating human plague cases.[184] Alternate regimens recommended by the Working Group on Civilian Biodefense include doxycycline (100 mg IV twice daily or 200 mg IV once daily), ciprofloxacin (400 mg IV twice daily) or chloramphenicol (25 mg/kg IV as a loading dose, followed by 60 mg/kg/d in four divided doses).[79]

Recently, a randomized, comparative, open label clinical trial comparing monotherapy with gentamicin or doxycycline for treating plague was conducted in Tanzania.[185] Thirty-five plague patients received gentamicin (2.5 mg/kg IM every 12 hours for 7 days) and 30 patients received doxycycline (100 mg [adults] and 2.2 mg/kg [children] orally every 12 hours for 7 days). Both gentamicin and doxycycline were found to be effective therapies for adult and pediatric plague, resulting in favorable response rates of 94% and 97% for gentamicin and doxycycline, respectively. [185] The three deaths occurred on the first or second day of treatment when disease was thought to be too advanced to respond to antibiotics. However, in this study a paucity of pneumonic plague cases existed, and the authors also recommended the use of a loading dose when using doxycycline (200 mg every 12 hours for 72 hours before completing the therapy with the conventional dose of 100 mg every 12 hours [or 200 mg every 24 hours]) to ensure early attainment of high-tissue concentrations of doxycycline in the face of a life-threatening infection.[186]

Chloramphenicol is indicated for conditions in which high tissue penetration is important, such as plague meningitis, pleuritis, or myocarditis, but it can cause bone marrow suppression. It can be used separately or combined with an aminoglycoside. In pregnant women, the preferred choice is gentamicin with doxycycline or ciprofloxacin as alternatives, and streptomycin should be avoided if possible.[79] The treatment of choice for plague in children is streptomycin or gentamicin. The Working Group on Civilian Biodefense recommended doxycycline, ciprofloxacin, or chloramphenicol as alternatives.[79] Chloramphenicol should not given to children younger than 2 years old because of the risk of grey baby syndrome.

In 2013, the Food and Drug Administration approved the fluoroquinolone antibiotic levofloxacin for treating patients with plague. This approval was based on the agency's Animal Efficacy Rule, which allows studies from animals to be used in situations where it is not feasible or ethical to conduct clinical trials in humans. In this study, 24 African green monkeys who had been challenged with aerosolized *Y pestis* of the CO92 strain received either placebo or levofloxacin at a dose equivalent to 500 mg IV every 24 hours for 10 days. Treatment was begun after the fever developed. Sixteen of 17 levofloxacin treated animals survived as compared with none of the seven control animals, which all died with 5 days of exposure. The one treated animal that died was euthanized because of vomiting and inability to retain food; blood cultures 2 to 4 days before death were negative.[80]

The Working Group on Civilian Biodefense has also proposed recommendations for antibiotic therapy in a mass casualty setting and for postexposure prophylaxis. Because IV or IM therapy may not be possible in these situations, oral therapy preferably with doxycycline or ciprofloxacin is recommended.[79] Levofloxacin, 500 mg once a day for 10 days, is also Food and Drug Administration- approved for postexposure prophylaxis[187] based on the Animal Efficacy Rule. If treated with antibiotics, buboes typically recede in 10 to 14 days and do not require drainage. Patients are unlikely to survive primary pneumonic plague if antibiotic therapy is not initiated within 18 hours of symptom onset. Without treatment, mortality is 60% for bubonic plague and 100% for the pneumonic and septicemic forms.[169]

Prevention

All plague-control measures must include insecticide use, public health education, environmental sanitation to reduce sources of food and shelter for rodents, and perhaps reduction of rodent populations with chemicals such as cholecalciferol.[27,40] Fleas must always be targeted before rodents, because killing rodents may release massive amounts of infected fleas.[161] The

use of insecticides in rodent areas is effective because rodents pick up dust on their feet and carry it back to their nests, where they distribute it over their bodies via constant preening.[27]

Postexposure Prophylaxis

Asymptomatic individuals such as family members, healthcare providers, or other close contacts with persons with untreated pneumonic plague should receive antibiotic prophylaxis for 7 days. Close contact is defined as contact with a patient at distance less than 2 m.[79] Prophylaxis is also recommended for laboratory workers who have had an exposure to *Y pestis*. Doxycycline is the preferred antibiotic, given as 100 mg twice daily for 7 days. Levofloxacin or ciprofloxacin are alternatives. The Working Group for Civilian Biodefense recommends that people who develop fever or cough while receiving prophylaxis should seek prompt medical attention and begin parenteral antibiotic treatment.[79] Hospital personnel who are observing recommended isolation procedures do not require prophylactic therapy, nor do contacts of bubonic plague patients. However, people who were in the same environment and were potentially exposed to the same source of infection as the plague patients should be given prophylactic antibiotics. The CDC also recommends that prophylactic antibiotics be given to persons potentially exposed to the bites of infected fleas (during a plague outbreak, for example) or who have handled animals infected with the plague bacterium. In addition, previously vaccinated individuals should receive prophylactic antibiotics if they have been exposed to plague aerosols.

Natural antibiotic resistance is rare in *Y pestis*; however, a chilling report appeared in 1997 of a human isolate in Madagascar resistant to streptomycin, tetracycline, chloramphenicol, ampicillin, kanamycin, and sulfonamide. A transmissible plasmid, pIP1202, was responsible for the multidrug-resistant phenotype of this isolate, suggesting a potential for transfer to other *Y pestis* strains in nature.[188] This plasmid is closely related to transmissible multidrug-resistant plasmids of *Yersinia ruckeri* and *Salmonella enterica* serotype Newport SL254 identified in the United States.[189] More recently, a multidrug-resistant strain of *Y pestis* was isolated from a rodent in Mongolia.[190] Russian scientists have published descriptions of multidrug-resistant plague vaccine strains produced in the laboratory; these techniques could conceivably be used on virulent strains.[191] Ciprofloxacin-resistant isolates have been obtained in the laboratory from attenuated strains.[192] If *Y pestis* is used as a biological weapon, then antibiotic resistance is a possibility; the stability and transmissibility of the multidrug-resistant pIP1202 plasmid in *Y pestis* suggests that such a strain could be engineered in the laboratory via conjugation without modern molecular technologies.

Vaccination

While working in India in 1897, Ukrainian microbiologist Waldemar MW Haffkine developed the first plague vaccine consisting of killed whole cells. In 1942, Karl F Meyer, DVM, began developing an immunogenic and less reactogenic vaccine for the US Army from an agar-grown, formalin-killed, suspension of virulent plague bacilli. This same procedure (with minor modifications) was used to prepare the licensed vaccine, Plague Vaccine USP, which was routinely given to military personnel stationed in Vietnam and other individuals such as field personnel working in plague-endemic areas with exposure to rats and fleas and laboratory personnel working with *Y pestis*. However, this vaccine was discontinued by its manufacturers in 1999 and is no longer available. Although Plague Vaccine USP was effective in preventing or ameliorating bubonic disease, as seen by the low incidence of plague in US military personnel serving in Vietnam, data from animal studies suggest that this vaccine does not protect against pneumonic plague.[151,152,193–195]

The former Soviet Union and many other nations have traditionally focused on live attenuated vaccines, with tens of millions of humans receiving the live plague vaccine. Many investigators continue to believe that live attenuated vaccines are preferable to subunit vaccines. Live plague vaccines, usually derived from the attenuated EV76 strain, have been used alone and also successfully in a prime-boost strategy with subunit vaccines. Even the most recent reviews on plague vaccination continue to revisit the appeal of live attenuated vaccines.[196,197]

Two new plague vaccine candidates that use the F1 and V antigens of *Y pestis* have been developed. F1, a capsular antigen of *Y pestis*, appears to prevent phagocytosis of plague bacilli, whereas V antigen has a key role in the translocation of the cytotoxic Yops into host cells, as well as stimulating the production of immunosuppressive cytokines. US Army Medical Research Institute of Infectious Diseases scientists developed the first vaccine, F1-V, which consists of a recombinant fusion protein expressing F1 and V antigens (F1-V).[198] Porton Down, the biodefense laboratory in the United Kingdom, developed a similar candidate that is a recombinant protein-based vaccine consisting of two separate proteins, F1 and V.[199] The separate proteins are then combined, two parts F1 to one part V, to form a subunit vaccine.

The F1-V vaccine, which has been shown to protect African green monkeys from pneumonic plague,[198] is currently in advanced development by the Department of Defense's Joint Project Manager Medical Countermeasures, a component of the Joint Program Executive Office for Chemical and Biological Defense. The Joint Project Manager Medical Countermeasures facilitates the advanced development and acquisition of medical countermeasures and systems to enhance the US biodefense response capability.

SUMMARY

Plague is a zoonotic infection caused by the gram-negative bacillus *Y pestis*. Plague is maintained in nature, predominately in urban and sylvatic rodents and flea vectors. Humans are not necessary for the persistence of the organism, and they acquire the disease from animal fleas, contact with infected animals, or, rarely, from other humans via aerosol or direct contact with infected secretions. Healthcare providers must understand the typical way in which humans contract plague in nature to differentiate endemic disease from plague used in biological warfare. First, a die-off of the mammalian reservoir that harbors bacteria-infected fleas will occur. Second, troops who have been in close proximity to such infected mammals will become infected and typically develop the bubonic form of the disease. By contrast, in the most likely biological warfare scenario, plague would spread via aerosol and result in primary pneumonic plague cases. Person-to-person spread of fulminant pneumonia, characterized by blood-tinged sputum, would then ensue. If, however, an enemy force released fleas infected with *Y pestis*, the soldiers would present with classic bubonic plague before a die-off in the local mammalian reservoir occurred, although such a die-off may be possible later if the introduced strain of *Y pestis* succeeded in proliferating among local rodent populations.

The most common form of the disease is bubonic plague, characterized by painful lymphadenopathy and severe constitutional symptoms of fever, chills, and headache. Septicemic plague without localized lymphadenopathy occurs less commonly and is difficult to diagnose. Secondary pneumonia may follow either the bubonic or the septicemic form. Primary pneumonic plague is spread by airborne transmission, when infectious respiratory droplets from an infected human or animal are inhaled or a person inhales an aerosol released as the result of biological weapon attack.

Diagnosis is established by isolating the organism from blood or other tissues. Rapid diagnosis may be made with fluorescent antibody stains of sputum or tissue specimens or detection of F1 antigen in serum. Patients should be isolated and treated with aminoglycosides. Chloramphenicol should be added when meningitis is suspected or shock is present. Although the licensed, killed, whole-cell vaccine is no longer available, a new vaccine that appears to protect against pneumonic plague is in advanced development.

Acknowledgments

We thank Andrea Stossel and Laura Kalinyak for assistance with collecting references for this chapter and the reviewers for their thoughtful comments. In addition, Dr Kenneth Gage provided valuable insight into the ecology of plague. This chapter is dedicated to long-time plague researchers Lieutenant Colonel Dan C Cavanaugh and Lieutenant Colonel John D Marshall, who made outstanding and enduring contributions to public health and military medicine in their careers as both Army officers and as civil servants.

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Chapter 11

TULAREMIA

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INTRODUCTION

Tularemia is a life-threatening, debilitating disease caused by infection with the bacterium *Francisella tularensis*. This bacterium is one of the most infectious microorganisms known and poses a substantial threat as a potential biological weapon.[1] Both the United States and the former Soviet Union developed weaponized *F tularensis* during the Cold War.[1,2] The Japanese experimented with *F tularensis* as a biological weapon,[3] but no documentation shows that it was deliberately used as a biological weapon. There is also speculation that the former Soviet Union used *F tularensis* as a biological weapon against German troops in the Battle of Stalingrad during World War II.[2] However, other authors suggest that natural causes, as opposed to an intentional release, were responsible for the tularemia epidemic that occurred in both armies during this battle.[4] There was similar speculation that *F tularensis* was used as a biological weapon by Serbia during the Kosovo conflict, although subsequent epidemiological investigations suggest that the observed cases were not caused by an intentional release.[5,6]

Given its highly pathogenic nature, low infectious dose, and ability to infect via aerosol, *F tularensis* is classified by the US Department of Health and Human Services as a tier 1 select agent. This classification is reserved for those pathogens deemed to pose the highest risk for intentional misuse. The nonspecific disease presentation of tularemia, the high morbidity, the significant mortality if untreated, and the limited ability to obtain a rapid diagnosis are other characteristics that contribute to the effectiveness of *F tularensis* as a potential biological weapon. Although tularemia responds to antibiotic therapy, the intentional use of a genetically modified antibiotic-resistant strain could make these countermeasures ineffective.

INFECTIOUS AGENT

Infection associated with *F tularensis* was first identified in Tulare County, California, where an epidemic disease outbreak resembling plague occurred in ground squirrels in 1911. McCoy and Chapin successfully cultured the causative agent and named it *Bacterium tularense*.[7] Subsequently, Wherry and Lamb identified this pathogen as the cause of conjunctival ulcers in a 22-year-old man.[8] Edward Francis' pioneering work significantly increased the understanding of human disease associated with this pathogen in the early 20th century. He described the clinical syndromes associated with *Francisella* infection and named it "tularemia."[9] *F tularensis* was formerly included in both the *Pasteurella* and the *Brucella* genera. However, as mounting scientific data supported the creation of a new genus for this remarkable pathogen, this bacterium was assigned to its own genus and the name *Francisella* was proposed in tribute to Edward Francis.[10]

F tularensis is an aerobic, nonmotile bacterium and member of the *Gammaproteobacteria*. By Gram stain, it appears as a small (approximately 0.2–0.5 μm by 0.7–1.0 μm),[11] faintly staining coccobacillus (Figure 11-1). *F tularensis* is considered to have four subspecies: (1) *tularensis*, (2) *holarctica*, (3) *mediasiatica*, and (4) *novicida*.[12] *F tularensis* subspecies *tularensis*, also known as type A (or biovar A), occurs predominantly in North America and is the most virulent subspecies in both animals and humans. This subspecies was recently divided into A.I. and A.II. subpopulations based on extensive genotyping of isolates. Subpopulation A.I. causes disease in the central United States, and subpopulation A.II. is found mostly in the western United States.[13] *F tularensis* subspecies *holarctica* (formerly described as *palearctica*), also known as type B (or biovar B), is found throughout the Northern Hemisphere. *F tularensis* subspecies *holarctica* typically causes a less clinically severe disease than subspecies *tularensis*, but has been documented to cause bacteremia in immunocompetent individuals.[14,15] Before antibiotics, *F tularensis* subspecies *tularensis* resulted in 5% to 57% mortality, yet *F tularensis* subspecies *holarctica* was rarely fatal.[15] *F tularensis* subspecies *mediasiatica* has

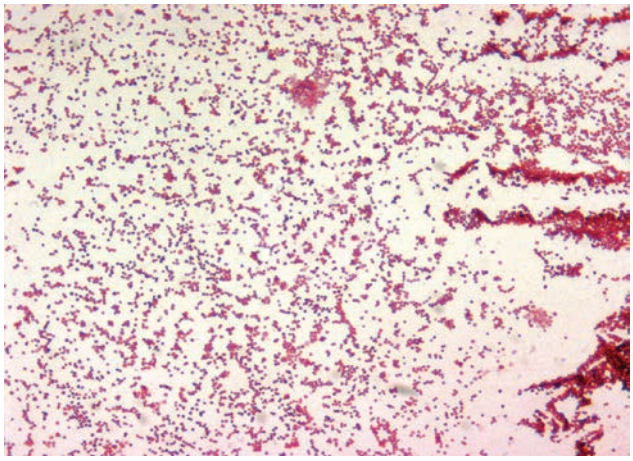


Figure 11-1. Gram's stain of *Francisella tularensis*.
Photograph: Courtesy of Dr Larry Stauffer, Oregon State Public Health Laboratories, Centers for Disease Control and Prevention, Atlanta, Georgia, Public Health Image Library, Image 1904.

been isolated in the central Asian republics of the former Soviet Union, and it appears to be substantially less virulent in a rabbit model compared to *F tularensis* subspecies *tularensis*.[16,17]

The clinical significance of this subspecies is not known. *F tularensis* subspecies *novicida*, also referred to as *F novicida*, is believed to be avirulent in healthy humans. Reported cases associated with this subspecies usually involve patients with other underlying health conditions.[18] The four subspecies can be distinguished using biochemical tests and genetic analysis. Another closely related species, *Francisella philomiragia*, has also been described as a human pathogen.[19,20] However, similar to *F tularensis* subspecies *novicida*, infections attributed to *F philomiragia* were typically found in patients with underlying health conditions.[19,20]

THE CLINICAL DISEASE

Tularemia is an infection with protean clinical manifestations. Healthcare providers need to understand the range of possible presentations of tularemia to use diagnostic testing and antibiotic therapy appropriately for these infections. Most cases of naturally occurring tularemia are ulceroglandular disease, involving an ulcer at the inoculation site and regional lymphadenopathy. Variations of ulceroglandular disease associated with different inoculation sites include ocular (oculoglandular) and oropharyngeal disease. Occasionally patients with tularemia present with a nonspecific febrile systemic illness (typhoidal tularemia) without evidence of a primary inoculation site. Pulmonary disease from *F tularensis* can occur naturally (pneumonic tularemia), but is uncommon and should raise suspicion of a biological attack, particularly if the cause is not readily discernable and significant numbers of cases are diagnosed. Because of the threat of this microorganism as a biological weapon, clusters of cases in a population or geographic area not accustomed to tularemia outbreaks should trigger consideration for further investigation.[21] Rotz et al provide criteria for determining the likelihood that a tularemia outbreak is caused by intentional use of *F tularensis* as a biological weapon.[21] A tularemia outbreak in US military personnel deployed to a nonendemic environment would be one example of an incident that should be investigated. An investigation should yield the likely cause of the outbreak, which could be varied (exposure to infected animals, arthropod borne, etc). By determining the source of the outbreak, it may be possible to implement control measures, such as water treatment or use of an alternative water supply if the outbreak is traced to a waterborne source.

Epidemiology

F tularensis subspecies *tularensis* (type A) is the most virulent subspecies and found predominantly in North America. This subspecies has recently been genetically subdivided into two subpopulations, A.I. and A.II. The subpopulations are distinct in mortality rates, geographic distribution, transmission vectors, and hosts.[22–27] *F tularensis* subspecies *holarctica* (type B), which is found throughout the Northern Hemisphere, is associated with a milder form of disease. In the United States, 90 to 154 cases of tularemia have been reported yearly from 2001 to 2010, according to the Centers for Disease Control and Prevention.[28] More than half of all cases reported came from Arkansas, Missouri, South Dakota, and Oklahoma, where the foci of infection are well established. Every state except Hawaii has reported cases of tularemia.

Human outbreaks, which are often preceded by animal outbreaks, are seasonal, with the highest incidence in late spring, summer, and autumn.[28,29] *F tularensis* has been detected in more than 100 mammalian species and several arthropods.[30] *F tularensis* can be transmitted to humans by direct contact with infected animals or their tissues, ingestion of undercooked infected meat or contaminated water, animal bites or scratches, arthropod bites, and inhalation of an aerosol or contaminated dust. However, human-to-human transmission has not been described. *F tularensis* is unique in its ability to adapt to a wide range of environmental, host, and vector conditions, and it can be categorized into two distinct transmission cycles involving different hosts and arthropod vectors. The cycle of disease is commonly associated with a subspecies, with type A commonly associated with the terrestrial cycle and type B commonly associated with the aquatic cycle.[23,27,31,32] The human clinical syndromes can be classified by the portal of entry.

Direct Contact

In 1914, a meat cutter with oculoglandular disease, manifested by conjunctival ulcers and preauricular lymphadenopathy, had the first microbiologically proven human tularemia case reported.[8] An early review of tularemia established that a majority of human cases (368 of 488, or 75%) in North America resulted from dressing and eating wild rabbits.[9] Other wild mammals may potentially serve as sources for tularemia transmission from direct contact, such as wild prairie dogs that are captured and sold as pets.[33]

Food and Water Ingestion

Tularemia can also be contracted by eating meat from infected animals[9] or food contaminated by infected animals.[34] Water can also become contaminated from animals infected with tularemia and cause human infection. From March through April 1982, 49 cases of oropharyngeal tularemia were identified in Sansepolcro, Italy.[3] The case distribution in this city suggested that a water system was the source. The infected individuals had consumed unchlorinated water, and a dead rabbit from which *F tularensis* was isolated was found nearby.[35] Waterborne transmission of ulceroglandular tularemia also occurred during a Spanish outbreak among 19 persons who had contact with river-caught crayfish.[36] The crayfish appear to have served as mechanical vectors, but some evidence suggests a potential role as hosts.[23,36] Contaminated water may have also contributed to recent outbreaks of oropharyngeal tularemia in Turkey[37] and Bulgaria.[34] It is unclear how *F tularensis* survives in water, but it may be linked to its ability to survive in certain protozoa species, such as *Acanthamoeba castellanii*.[38]

Mammalian Bites and Arthropod Vectors

Mammalian bites are another source of *F tularensis* transmission to humans. Instances of transmission from the bites or scratch of a cat, coyote, ground squirrel, and a hog to humans were documented more than 80 years ago.[9] In April 2004, a 3-year-old boy from Denver, Colorado, contracted tularemia from a hamster bite, providing evidence of disease transmission from these pets.[39] Transmission of *F tularensis* by the bites of ticks and flies is also well documented.[11] *Dermacentor* species ticks (dog ticks) are important vectors in areas where enzootic transmission occurs in North America[40] and Europe.[41] *Ixodes* species ticks may also contribute to *F tularensis* transmission.[42] In Utah during the summer of 1971, 28 of 39 tularemia cases were contracted from deerfly (*Chrysops discalis*) bites.[43] An epidemic of 121 tularemia cases (115 ulceroglandular) in Siberia from July through August 1941 may have resulted from transmission of *F tularensis* by mosquitoes, midges (*Chironomidae*), and small flies (*Similia*).[44]

Aerosol Transmission

The largest recorded pneumonic tularemia outbreak occurred in Sweden during the winter of 1966 through 1967, when 676 cases were reported.[45] Most of the cases occurred among the farming population, 71% among adults older than 45 years and 63% among men. The hundreds of pneumonic cases likely resulted from contact with hay and dust contaminated by voles infected with tularemia. *F tularensis* was later isolated from the dead rodents found in barns, as well as from vole feces and hay.

In the summer of 2000, an outbreak of primary pneumonic tularemia occurred in Martha's Vineyard, Massachusetts.[46] Fifteen confirmed tularemia cases were identified, 11 of which were the pneumonic form of tularemia. One 43-year-old man died of primary pneumonic tularemia. Epidemiological analysis revealed that using a lawn mower or brush cutter was significantly associated with illness in the 2 weeks before presentation of this case.[47] Feldman et al proposed that in Martha's Vineyard, *F tularensis* was shed in animal excreta, persisted in the environment, and was transmitted to humans after mechanical aerosolization by mower or brush cutter and subsequent inhalation.[47] The strong epidemiological link with grass cutting adds plausibility to this explanation.[48] A seroprevalence survey conducted in 2001 in Martha's Vineyard demonstrated that landscapers were more likely to have antibodies to *F tularensis* than nonlandscapers, suggesting an increased occupational risk for tularemia.[47]

The only other previously reported outbreak of pneumonic tularemia in the United States occurred in Martha's Vineyard during the summer of 1978.[49] In a single week, seven persons who stayed together in a vacation cottage eventually developed typhoidal tularemia. A search for additional cases on the island uncovered six other tularemia cases (five typhoidal and one ulceroglandular). No confirmed source for the disease exposure was discovered. Tularemia had been reported sporadically since the introduction of rabbits to Martha's Vineyard in the 1930s,[49] and pneumonic tularemia was initially reported in Massachusetts in 1947.[50]

Tularemia in an Unusual Setting

Some tularemia cases have occurred in geographic areas where the disease has never been reported. An orienteering contest on an isolated Swedish island in 2000 resulted in two cases of ulceroglandular tularemia.[51] These cases were theorized to have occurred from contact with migratory birds carrying the microorganism.

The social disruption caused by war also has been linked to tularemia outbreaks. During World War II, an outbreak of more than 100,000 tularemia cases occurred in the former Soviet Union,[4] and outbreaks with hundreds of cases after the war occurred in Austria and France.[52] Outbreaks of zoonoses during war since that time have led to speculation that these epidemics were purposefully caused. For example, no tularemia cases had been reported from Kosovo between 1974

and 1999, and tularemia was not previously recognized endemically or enzootically in the Balkan countries.[5] However, after a decade of warfare, an outbreak of more than 900 suspected tularemia cases occurred in Kosovo during 1999 and 2000, leading researchers to investigate claims of use of this agent as a biological weapon by the Serbs against the Albanian inhabitants of the country.[56] The Kosovo outbreak and subsequent investigation are described in detail in chapter 2, Epidemiology of Biowarfare and Bioterrorism.

Laboratory-acquired Tularemia

Soon after the discovery of *F tularensis* as a pathogen, cases of laboratory-acquired infection were recognized. Edward Francis observed that many laboratory personnel working with the pathogen, including himself, became infected.[9] Six tularemia cases occurred during US Public Health Service laboratory investigations of tularemia outbreaks from 1919 through 1921.[53] Tularemia is the third most commonly acquired laboratory infection,[54] and recent laboratory-acquired infections of tularemia emphasize the laboratory hazard that this organism presents.[55] Because of the extreme infectivity of this microorganism, investigators of a 2000 outbreak in Kosovo chose not to culture the organisms from patients, but instead relied on empirical clinical evidence of tularemia cases.

Pathogenesis

One of the remarkable attributes of *F tularensis* is its low infectious dose. As few as 10 organisms can produce clinical disease in healthy human volunteers when administered by either subcutaneous injection or by aerosol exposure.[56,57] Research aimed at elucidating the unique characteristics that permit this organism to cause disease at such low numbers revealed that *F tularensis* boasts a variety of mechanisms to not only evade host defenses, but also to modulate them to survive and proliferate within its host.

F tularensis, which is an intracellular pathogen, is known to survive and replicate within a wide variety of cells including professional phagocytic cells, such as macrophages. To gain entry into these cells, *F tularensis* can efficiently use multiple receptors including the mannose receptor, FcγR, and complement receptor 3. Interestingly, a recent study using a fully virulent type A strain showed that entry of opsonized bacteria into human macrophages via complement receptor 3 suppressed the Toll-like receptor 2-dependent proinflammatory responses.[58] Bacterial entry through the mannose receptor resulted in rapid phagosomal escape and prolific cytosolic replication.[59] These findings indicate that *Francisella* has evolved to use multiple entry pathways to enhance its ability to replicate in the intracellular environment.

Once inside the macrophage, *Francisella* can avoid the bactericidal activity of reactive oxygen species and nitrogen species through expression of enzymes including bacterial acid phosphatases (Acp), superoxide dismutases (Sod), and catalase enzymes (Kat).[60–65] Inhibition of these host defense mechanisms promotes bacterial virulence, as *F tularensis* live vaccine strain (LVS) mutants deficient in expression of SodB, SodC, or KatG are highly attenuated in mouse models of tularemia.[60,61,63] Phagosomal acidification is another host defense mechanism designed to restrict growth of bacterial pathogens. However, both *F tularensis* type A and B stains can inhibit acidification of the phagosome and subsequently escape from the phagosome, and reside in the macrophage cytoplasm.[66,67] The ability of *Francisella* to escape into the cytosol is in part dependent on proteins encoded on the *Francisella* pathogenicity island (FPI). Nano et al first described the FPI in 2004 and subsequently most genes contained within the FPI have been linked to virulence.[68] The FPI also contains genes that encode for a putative type VI secretion system that is required for phagosomal escape and virulence.[69,70] *IglC*, a 23-kDa protein, is believed to be both a core component and secreted effector of the T6SS. *IglC* has been implicated not only in phagosomal escape but also in influencing Toll-like receptor-4 signal transduction.[71–75] Regulation of the FPI is controlled by the *MglA* transcriptional regulator, which responds to various cues and in turn influences expression of more than 100 genes, including several other virulence factors.[76]

Once *Francisella* reaches the cytoplasm, replication begins slowly, but eventually large numbers of organisms can be found within a single macrophage.[73,77,78] Although *F tularensis* may initially delay apoptosis (programmed cell death) of the macrophage, the organism eventually induces apoptosis through mechanisms similar to intrinsic cellular signals.[79] This strategy to escape the macrophage may help shield *Francisella* from host immune surveillance mechanisms.

Another survival mechanism of *F tularensis* is the inhibition of Toll-like receptor signaling and cytokine secretion, as demonstrated in experiments with murine macrophages and the LVS of *F tularensis*.[75] Avoidance of Toll-like receptor signaling inhibits the typical robust innate immune response that is active in eliminating typical bacterial pathogens. The early innate immune response to *F tularensis* involves intracellular killing of the pathogen by the macrophages and proinflammatory cytokine secretion. Murine experiments have demonstrated the importance

of an effective early cytokine response. Interferon-γ-deficient mice die from sublethal doses of LVS,[80] and tumor necrosis factor-α is at least as important as interferon-γ for control of *F tularensis* infection.[81,82] The host defense within macrophages appears to be crucial at controlling infection by *F tularensis*. In human monocytes/macrophages, the LVS strain and *F novicida* induced the processing and release of interleukin (IL)-1β, an essential component of the inflammatory immune response.[83] However, killed bacteria did not induce this response, but did induce the early phases required for IL-1β, such as mRNA transcription. This suggests that only live *Francisella* can escape from the phagosome, and thus trigger the function of caspase-1, which converts the precursor of IL-1β to its active form. In mice deficient in caspase-1 as well as ASC, an adaptor protein involved in the assembly of inflammasome complexes, substantially higher bacterial loads were observed, as well as early mortality, compared to normal mice.[84] Neutrophils perform an important function in limiting the spread of *F tularensis* after inoculation. Experiments have demonstrated that neutrophils can kill *F tularensis*,[85] and mice depleted of neutrophils appear more susceptible to infection with *F tularensis* LVS.[86]

The late adaptive immune response to *F tularensis* requires an intact cell-mediated immune system, particularly in resolving the initial infection and in producing long-term immunity.[87] There is no clear immunodominant epitope on any one *F tularensis* virulence protein that stimulates the required cell-mediated response; however, studies have demonstrated that multiple protein/peptides may be required to produce a sufficient response.[88] Vaccination with *F tularensis* LVS appears to produce a long-term memory T-cell response (as measured by lymphocyte stimulation),[89] but it is unclear what degree of long-term protection is conferred by this response. Both $CD4^+$ and $CD8^+$ lymphocytes are required for an effective cell-mediated response to *F tularensis*.[80] The protective memory response is dependent on a robust proinflammatory cellular response, because administration of anti-interferon-γ and antitumor necrosis factor-α antibodies to previously vaccinated mice dramatically lowers the lethal infective intradermal dose of *F tularensis*.[82] This memory response initially appears 2 to 4 weeks after initial infection,[90–92] and it can remain detectable for many years.[89,93]

The importance of humoral immunity in the defense against tularemia is not completely understood, but it appears that the humoral response by itself provides little or no value in protecting the host.[94] When laboratory workers received a formalin-killed whole-cell vaccine developed by Foshay et al,[95] a strong humoral response was elicited but was not protective against cutaneous[58] or respiratory[57] challenge. The failure of this vaccine suggested that the formalin inactivation procedures destroyed some of the essential protective antigens or that these protective antigens were not expressed in vitro. A persistent humoral response does develop during human infection and after vaccination. Waag et al reported that sera from five of nine vaccinees resulted in Western blot banding profiles that were identical to *F tularensis* lipopolysaccharide.[90] Investigations focused on identifying protective antigens are ongoing, particularly in animal models.[12] Unfortunately, the antigens that induce humoral immunity appear to be different than antigens inducing cell-mediated immunity, making determinations of the most immunogenic antigen challenging.[94] The ultimate goal of these investigations is to optimize the cell-mediated immune response to *F tularensis*, thereby suggesting improvements to prophylactic and therapeutic strategies.

The lipopolysaccharide structure of many gram-negative pathogens elicits a profound proinflammatory immune response, which can lead to the clinical manifestations of septic shock.[96] However, although *F tularensis* lipopolysaccharide can elicit a strong humoral response, it does not induce significant tumor necrosis factor-α and nitric oxide production in macrophages or IL-1 from polymorphonuclear cells,[97] in contrast to lipopolysaccharide from other gram-negative pathogens. Additionally, the lipopolysaccharide of *F tularensis* is structurally different in composition than typical gram-negative pathogens, which is believed to result in the poor Toll-like receptor 4 stimulation observed in type A and type B strains.[98] The O-antigen of *Francisella* has also been shown to be required for virulence. The ability of *Francisella* to avoid complement mediated killing is dependent on the presence of O-antigen as *F tularensis* mutants deficient in O-antigen expression are more sensitive to complement.[98] O-antigen was required for virulence of *F tularensis* in mice[99] and also played a role in cytosolic survival by avoiding autophagy.[100]

Clinical Manifestations

Tularemia has a diversity of clinical presentations, and it is likely that many cases are unrecognized, especially because of the diagnostic challenges associated with this infection.[101] The disease manifestations of tularemia have been ascribed to at least 10 different clinical categories (ulceroglandular, glandular, oculoglandular, oropharyngeal, enteric, gastrointestinal, typhoidal, respiratory, pneumonic, and septic). Symptoms overlap among these categories.[102] Alternatively,

Evans' review of 88 tularemia cases more than 30 years ago describes two syndromes based on clinical signs (ulceroglandular or typhoidal), portal of entry, and disease prognosis. This categorization simplifies this often confusing nomenclature, while emphasizing the obscure but potentially fatal typhoidal presentation, and may also reflect differences in host response to *F tularensis* infection.[103] With ulceroglandular tularemia, there is a vigorous inflammatory reaction, pneumonia is uncommon, and the patient rarely succumbs from infection. Typhoidal tularemia presents with few localizing manifestations, pneumonia is common, and mortality is higher in the absence of antimicrobial therapy.[11,104]

Typhoidal tularemia (15%–25% of cases) primarily occurs after infectious aerosol inhalation, but may also result from an intradermal or gastrointestinal infection.[11,104] The disease presents as a nonspecific syndrome with an abrupt onset of fever (38°C to 40°C), headache, malaise, myalgias, and prostration, but without lymphadenopathy.[11] Lymph nodes are less than 1 cm in diameter, and no skin or mucous membrane lesions are present. Nausea, vomiting, diarrhea, and abdominal pain may also occur. Mortality is greater with pneumonia.[11] Case fatality rates are approximately 35% in untreated naturally acquired typhoidal tularemia.[102] Untreated tularemia survivors may have persistent symptoms for weeks or months with progressive debilitation.[102]

Ulceroglandular tularemia (75%–85% of naturally occurring disease) most often occurs through mucous membrane or skin inoculation with blood or tissue fluids of infected animals.[104] Clinical symptoms in cases of ulceroglandular tularemia typically appear after an incubation period of 3 to 6 days. The lesions present on the skin or mucous membranes (including conjunctiva, oropharynx, etc) and lymph nodes are greater than 1 cm in diameter.[11] This form of the disease is characterized by sudden onset of fever (85% of cases), chills (52% of cases), headache (45% of cases), cough (38% of cases), and myalgias (31% of cases), along with the emergence of a painful papule at the inoculation site.[104] The fever may be associated with pulse-temperature disassociation (42% of cases in one series)[104] where the pulse increases fewer than 10 beats per minute per 1°F increase in temperature above normal. However, this finding is not specific for tularemia. Other nonspecific complaints include chest pain, vomiting, arthralgia, sore throat, abdominal pain, diarrhea, dysuria, back pain, and nuchal rigidity.[102,104] A rapid progression occurs at the site of inoculation in the untreated patient, with pustule formation leading to a painful ulcer, accompanied by regional painful lymphadenopathy. A persistent ulcer is the hallmark of ulceroglandular tularemia. Ulcers generally range in size from 0.4 cm to 3.0 cm and occasionally have raised borders.

The location of the lesion may provide an indirect clue as to the route of exposure: inoculation from an arthropod vector, such as a tick, is more likely on the lower extremities, and exposure to a mammal with tularemia tends to cause lesions on the upper extremities.[104] Lesions are typically associated with regional lymphadenopathy, and a lack of lymphadenopathy may suggest another etiologic agent.[104] Enlarged lymph nodes can occur singly, in groups, or enlarged in a sequential fashion along the lymphatic tracts (sporotrichoid pattern). The lymph node is typically painful and may precede, occur simultaneously, or follow the appearance of the cutaneous ulcer in ulceroglandular disease.[102] Enlarged lymph nodes may become fluctuant and spontaneously drain. If untreated, these fluctuant lymph nodes may persist for months or years.[102] The ulceroglandular form in the eye (oculoglandular) presents with ocular erythema and exudative conjunctivitis as key distinguishing features. The mechanism of exposure is usually from contact with infected mammals.

One case report describes infection after tick removal; the tick contents were inadvertently inoculated into the eye.[105] Food and water contamination can also lead to oculoglandular infection.[34] In one series pharyngitis was observed in 24% of patients with tularemia.[104] Possible findings on examination include erythema, exudates, petechiae, hemorrhage, or ulceration. Other findings may include retropharyngeal abscess or suppuration of the regional lymph nodes. Severe exudative pharyngitis suggests ingestion of contaminated food or water as the likely source of infection. The appearance of pharyngitis may be linked to lower respiratory tract disease, or possibly to ingestion as the route of exposure. Oropharyngeal signs and symptoms and cervical adenitis have been the primary manifestation of recent outbreaks in Turkey (83% of cases)[37] and Bulgaria (89% of cases),[34] and these outbreaks appear to be associated with a contaminated water source.

The overall incidence of symptoms of lower respiratory tract disease in patients with tularemia is high, ranging from 47% to 94%.[52,104] These percentages are influenced by the route of exposure and the diagnostic approach to a patient with tularemia. The routine use of chest radiographs increases the likelihood of detecting mild or asymptomatic respiratory infections. Additionally, case series may only involve patients who are hospitalized, or receive a thorough evaluation, and may not include milder case presentations. Pneumonic tularemia can result from cases

of ulceroglandular tularemia through hematogenous spread, with an onset ranging from a few days to months after the appearance of initial nonpulmonary symptoms.[52] Approximately 30% of patients with ulceroglandular disease and 80% of patients with typhoidal tularemia also have pulmonary signs and symptoms consistent with pneumonia.[104] Pneumonic tularemia can also occur from direct inhalation of the organism, which has been demonstrated in human experimental models.[56,106] In experimental infections of humans, cases were characterized by abrupt onset of fever, headache, sore throat, malaise, myalgias, coryza, and cough, which was typically nonproductive.[106] Chest radiographic findings in pneumonic tularemia are highly variable and nonspecific[107] because they can mimic findings associated with other clinical syndromes such as bacterial pneumonias, tuberculosis, lymphoma, or lung carcinoma.[108] Patients can have infiltrates consistent with pneumonia and hilar adenopathy. In patients with pneumonia, 15% have an associated pleural effusion. Other less common findings include interstitial infiltrates, cavitary lesions, and bronchopleural fistulas.

A pneumonic tularemia outbreak in Martha's Vineyard, Massachusetts, provides an instructive example of tularemia's diagnostic challenges. The index case was a Connecticut resident with a second home at Martha's Vineyard. His family physician in Connecticut empirically treated this case of "summer pneumonia." Hospital clinicians in Martha's Vineyard noticed the outbreak more than a month later while searching for the cause of another pneumonic summer illness.[46,109] After seeing news accounts of the Martha's Vineyard tularemia outbreak, the Connecticut man reported to Connecticut health authorities with a history of symptoms, exposure risk, and laboratory tests compatible with tularemia.

Other examples of pneumonic tularemia have presented as diagnostic challenges. In 1994, a California case of community-acquired pneumonia was recognized as typhoidal tularemia in a 78-year-old with an absence of any epidemiological association for the illness.[110] A decade earlier, of the 96 patients with tularemia presenting to a Veteran's Hospital in Arkansas, five had pneumonic tularemia.[111]

The clinical manifestations of typhoidal and septic forms of tularemia overlap. Septic tularemia can be considered the result of clinical progression of any of the other forms of tularemia to a state of septic shock. Typhoidal tularemia presents as a nonspecific febrile syndrome, with or without lymphadenopathy, that can lead to death if untreated.[108] This presentation mimics an extensive number of other disease entities, making the diagnosis challenging. A wide range of additional clinical manifestations has been described with all forms of tularemia, including pericarditis, enteritis, appendicitis, peritonitis, erythema nodosum, and meningitis.[101,104,112]

The laboratory findings with tularemia are nonspecific. Hemoglobin and platelet counts are typically normal, but anemia has been associated with disease. White blood cell counts are usually only mildly elevated, with no alteration in the normal cell differential.[104] Microscopic pyuria may be observed[104] and nonspecific inflammatory markers such as erythrocyte sedimentation rate and C-reactive protein can be elevated. One case series described tularemia associated with skeletal muscle abscesses, elevated creatine kinase, and rhabdomyolysis.[113] Nonspecific elevations of liver transaminases and alkaline phosphatase may be observed with tularemia. The cerebrospinal fluid is usually normal, but may have mildly abnormal glucose, protein, and cell counts.[104]

Untreated tularemia patients usually have a prolonged illness lasting for months. The disease can be fatal, although rarely in ulceroglandular tularemia with antibiotic intervention. Before the use of streptomycin for therapy, tularemia—particularly the typhoidal form—had a mortality rate of 33%.[102] No specific infection control practices are recommended for tularemia, other than universal precautions, because no documented cases of human-to-human transmission exist.[1] However, special precautions are needed for clinical microbiology laboratory workers because of the high incidence of laboratory-acquired infections[114] (see Issues for Laboratory Workers).

Diagnosis

The diagnosis of tularemia is difficult because the clinical presentations of the various forms are not specific and are consistent with several other syndromes. This nonspecific presentation combined with a low incidence rate may have the unintended consequence of excluding tularemia as a differential diagnosis. This situation is exemplified by a review of cases in Missouri, a known focal point of infection in the United States, where more than half of the documented tularemia infections were misdiagnosed as other infectious diseases.[115] Additionally, the diagnostic modalities available for isolation and identification of *F tularensis* have limitations. In a scenario in which *F tularensis* is used as a biological weapon, a rapid increase in pneumonic cases may be the initial clue implicating a biological weapon attack. In this scenario, either astute clinical judgment[116] or epidemiological syndromic surveillance[117] would be useful in detecting the attack.

Bacterial Culture Techniques

The diagnosis of tularemia by culture can be challenging because the organism grows poorly on routine culture medium. Although positive cultures have been obtained from the blood,[118–120] blood cultures are typically negative, even in cases of severe disease.[15] Similarly, cultures from ulcer sites, sputum, gastric washings, and pharyngeal and conjunctival exudates are also usually negative.[11] Occasionally, positive blood cultures have been observed in immunocompromised persons (infected with the less virulent subspecies *holarctica*), and recovery may be improved when blind subculture is conducted.[120]

F tularensis is difficult to grow using standard media and requires media supplemented with cysteine or other sources of sulfhydryl groups.[121] Chocolate agar, charcoal yeast extract agar, and Thayer-Martin agar support the growth of *F tularensis*. *F tularensis* colonies appear gray-white on chocolate or Thayer-Martin agar (Figure 11-2). The organism is optimally grown in a CO_2 incubator and tends to grow more slowly than bacteria routinely encountered in clinical practice typically taking 48 to 72 hours to discern. The fastidious growth characteristics of *F tularensis* can often make the diagnosis of tularemia difficult, particularly when only routine culture techniques are used. However, some strains of *F tularensis* (ie, *novicida* subspecies) do not have these fastidious growth requirements.[122]

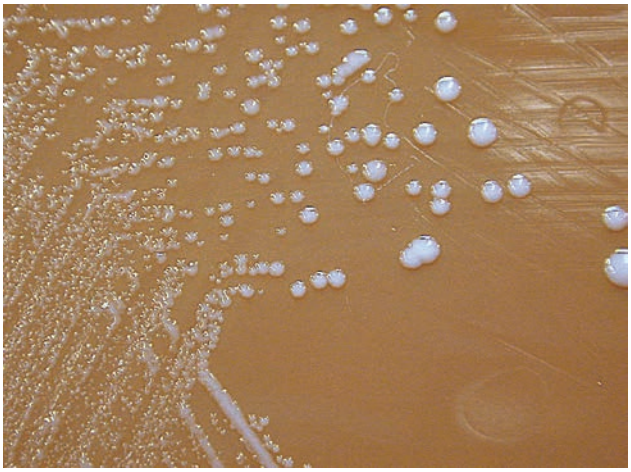


Figure 11-2. Chocolate agar plate of *Francisella tularensis*. Photograph: Courtesy of Dr Larry Stauffer, Oregon State Public Health Laboratories, Centers for Disease Control and Prevention, Atlanta, Georgia, Public Health Image Library, Image 1912.

When recovered from clinical specimens, the organism may be presumptively identified with traditional microbiology techniques and biochemical testing. Automated identification systems in microbiology laboratories should be avoided because they may create aerosols and often misidentify the pathogen. Presumptive or suspected *F tularensis* isolates should be referred to a specialized laboratory for definitive testing.

Serology

Traditionally, tularemia diagnosis has been based on serology, with a 4-fold rise in antibody titer as an acceptable diagnostic criterion. When using a microagglutination test, levels of antibody may be measurable within 1 week after infection, although significant levels usually appear in 2 weeks. An agglutination titer of greater than 1:160 tends to be specific for *F tularensis* infection. These criteria are used in a major case series on tularemia.[104]

The limitations of serologic diagnosis are as pertinent to tularemia as they are to other infections. This technique depends on obtaining acute and convalescent sera, which may not be practical, especially if the suspicion of tularemia is delayed because of a nonspecific presentation.[123] Antibodies to *F tularensis* may cross-react with other bacteria, such as *Brucella*, *Proteus*, and *Yersinia* species, which decreases the specificity of serology-based assays. Antibiotic therapy can blunt the serologic response, which could mask the convalescent rise in titer needed to confirm the diagnosis. Finally, antibody levels against *F tularensis* can persist for years, so distinguishing between acute and remote infection may be difficult. For all of these reasons, the development of better diagnostic capabilities for tularemia has become imperative.[1]

Rapid Diagnostic Methods

The most promising recent development in tularemia diagnosis has been the application of polymerase chain reaction (PCR) technology. *F tularensis* can be detected by standard PCR of the 16S rRNA gene[124,125] and the genus-specific *tul4* gene encoding a 17-kd membrane lipoprotein.[125–128] Other PCR assays have been designed to target *fopA*, a locus encoding an outer membrane protein.[126,129,130] PCR testing of tissue specimens has been performed with mouse models,[131] rabbit tissue,[132] and humans with ulceroglandular tularemia.[125,133] However, PCR as a diagnostic test has some limitations. The limit of detection of *F tularensis* in blood samples may be suboptimal because of the presence of PCR inhibitors[11] or other unknown con-

founding factors. Antigen-detection techniques have also been developed for *F tularensis*,[132,134] although extensive data on the specificity and sensitivity of these techniques have not been published. These techniques offer the potential of rapid detection, but have not been extensively used in human clinical case scenarios. Other assays to detect *F tularensis* have been studied, including capture enzyme-linked immunosorbent assays based on monoclonal antibodies specific for lipopolysaccharide and fluorescent antibody tests for detection in pathological samples.[31,132]

Treatment

Antibiotics usually provide curative therapy for tularemia, with resulting mortality rates of only 1% to 2.5%.[1,104] Mortality varies, depending on type of infection (ulceroglandular vs typhoidal), overall health of the infected individual, and rapidity after infection that antimicrobial therapy was initiated. Streptomycin has traditionally been used to treat tularemia, with individuals often demonstrating a clinical response within 48 hours of administration.[1,11,135] Relapses with streptomycin rarely occur. Gentamicin or other aminoglycosides are thought to be as effective as streptomycin,[136,137] but no controlled trials have been reported. Beta-lactam antibiotics such as ceftriaxone[136] are typically ineffective.

Antibiotics other than the aminoglycosides have been proposed for treating tularemia. Tetracycline and doxycycline are effective, but are associated with a higher relapse rate than the aminoglycosides.[1,123,136] Chloramphenicol is another alternative,[1] but it is rarely used in the United States. The fluoroquinolones offer an additional treatment option,[138–140] especially with the high bioavailability of oral preparations. Although extensive clinical data are lacking for the fluoroquinolones, one report of a tularemia outbreak resulting from *F tularensis* subspecies *holoarctica* in Spain noted a 5% failure rate for ciprofloxacin, compared to a 23% failure rate for streptomycin and 43% failure rate for doxycycline.[123] However, the number of patients treated with streptomycin in this study was 94, compared to only 22 being treated with ciprofloxacin. Although the clinical effectiveness with fluoroquinolones has been demonstrated in mild to moderate cases resulting from *F tularensis* subspecies *holoarctica*, in severe cases a combination with gentamicin has been recommended.[141]

However, there is limited experience using fluoroquinolones to treat tularemia disease due to the more virulent *F tularensis* subspecies *tularensis*, but

TABLE 11-1

ANTIBIOTICS FOR THE TREATMENT OF TULAREMIA*

Patient Group	Preferred Antibiotic	Dose	Alternate	Dose
Adults	Streptomycin	1 g IM twice daily	Doxycycline	100 mg IV twice daily
	Gentamicin*	5 mg/kg IM or IV once daily	Ciprofloxacin*	400 mg IV twice daily
			Chloramphenicol*	15 mg/kg IV four times a day
Children	Streptomycin	15 mg/kg IM twice daily	Doxycycline	If weight is >45 kg, 100 mg IV twice daily; if weight is <45 kg, 2.2 mg/kg IV twice daily
	Gentamicin*	2.5 mg/kg IM or IV three times daily	Ciprofloxacin*	15 mg/kg IV twice daily
			Chloramphenicol*	15 mg/kg IV four times daily
Pregnant Women	Gentamicin*	5 mg/kg IM or IV once daily	Doxycycline	100 mg IV twice daily
	Streptomycin	1 g IM twice daily	Ciprofloxacin†	400 mg IV twice daily

*Recommendations are from the Working Group on Civilian Biodefense, and assume a contained casualty setting. Recommendations would differ in a mass casualty scenario.
†Usage is not approved by the Food and Drug Administration.
IM: intramuscular.
IV: intravenous.
Data source: Dennis DT, Inglesby TV, Henderson DA, et al. Tularemia as a biological weapon: medical and public health management. *JAMA.* 2001;285:2763–2773.

it has been used successfully in a case that relapsed after doxycycline.[142] The use of combination antibiotic therapy has not been studied for severe tularemia cases, nor has the antimicrobial susceptibility of antibiotic-resistant strains been extensively studied. The treatment options are summarized in Table 11-1.

The general recommendations for length of therapy depend on the antibiotic used. Aminoglycosides and ciprofloxacin are thought to have a low incidence of relapse and, therefore, a course of 10 days is recommended.[1] For doxycycline and chloramphenicol, a longer course of 14 to 21 days is indicated.[1]

PROPHYLAXIS

Postexposure Prophylaxis

Recent consensus recommendations have addressed the issue of postexposure prophylaxis after the use of *F tularensis* in a biological attack.[1] These recommendations have suggested that antibiotics are indicated, especially if the exposure is thought to be recent. Data from human challenge models have suggested that tetracycline can be used to prevent infection after exposure.[143] In an experiment in which volunteers received tetracycline within 24 hours after airborne exposure to *F tularensis*, no tularemia symptoms were detected in 8 volunteers receiving 2 g per day for 14 days, or in 8 volunteers receiving 1 g per day for 28 days. In a group in the same experiment receiving 1 g per day for 15 days, 2 of 10 volunteers developed symptoms after therapy was discontinued. Therefore, if patients can be treated in the early incubation period, oral therapy with either ciprofloxacin or doxycycline (a compound closely related to tetracycline) for 14 days is suggested. However, if the exposure is not detected immediately and it is suspected that individuals were exposed more than a few days ago, a "fever watch" is recommended, involving self-monitoring for constitutional symptoms such as a fever or flu-like illness.[1] Individuals who develop these symptoms should be presumptively treated as if they had tularemia. Consensus statements for postexposure prophylaxis are described in Table 11-2.

TABLE 11-2

ANTIBIOTICS FOR POSTEXPOSURE PROPHYLAXIS*

Type of Patient	Preferred Antibiotic	Therapy
Adult	Doxycycline	100 mg orally twice daily
	Ciprofloxacin†	500 mg orally twice daily
Children	Doxycycline	If weight is >45 kg, 100 mg orally twice daily; if weight is <45 kg, 2.2 mg/kg orally twice daily
	Ciprofloxacin†	15 mg/kg orally twice daily
Pregnant women	Ciprofloxacin†	500 mg orally twice daily
	Doxycycline	100 mg orally twice daily

*Recommendations are from the Working Group on Civilian Biodefense.
†Usage is not approved by the Food and Drug Administration.
Data source: Dennis DT, Inglesby TV, Henderson DA, et al. Tularemia as a biological weapon: medical and public health management. *JAMA.* 2001;285:2763–2773.

Vaccination With Live Vaccine Strain

A live vaccine for *F tularensis* was first developed in the former Soviet Union in the 1930s and reportedly used to safely vaccinate millions of individuals.[144] This vaccine, developed from a type B strain, was transferred in 1956 to the United States,[145] where researchers Eigelsbach and Downs further characterized the strain designating it as the LVS of *F tularensis*. It is the only tularemia vaccine available in the United States and is currently in Food and Drug Administration Investigational New Drug status. This vaccine has been administered to thousands of recipients since the 1950s at the US Army Medical Research Institute of Infectious Diseases (USAMRIID). The vaccine is administered by a scarification process (similar to smallpox vaccination) to the volar surface of the forearm. A small papule forms initially, developing occasionally into a pustule and ulcer. Most vaccine recipients develop a minor scab, and few have systemic side effects. In human challenge studies, the vaccine protected against low to moderate-dose respiratory challenge and partially protected against high-dose respiratory challenge with virulent type A strains.[56,106] Alternative vaccine strategies have been the focus of considerable research, but none of these candidate vaccines are ready for human use.

F tularensis LVS has been studied extensively in mice, but significant differences exist in the immune response of mice to this type B strain and the immune response of humans to type A strains. LVS can be fatal in mice when administered as an intraperitoneal injection, yet it can confer protective immunity if given as an intradermal injection.[81] Intradermal administration of LVS can also protect mice from a lethal challenge dose

of virulent strains of *F tularensis*. Mice can be protected from the virulent form of *F tularensis* as early as 2 to 3 days after intradermal injection of LVS.[146] Injections of bacterial DNA (as unmethylated CpG motifs) can also confer a similar early protective response.[147] The prompt development of immunity after vaccination in mice suggests that the protective mechanisms are attributable to innate immunity[80] because an adaptive response requires more time to develop. It is unknown whether the vaccine in humans induces an early immune response that is protective. This type of early protection after vaccination would be useful in the military environment because unexposed soldiers may be rapidly protected from further intentional use of *F tularensis* as a weapon.

The correlates of immune response to vaccination have been suggested by prior investigations, but are not definitively established. Before the use of LVS, a killed *F tularensis* vaccine was used.[95] This vaccine was documented to elicit a serologic response, but was not protective. Markers of cell-mediated immunity, such as delayed-type hypersensitivity testing, have also been correlated with protection after vaccination.[94]

The LVS tularemia vaccine is offered at the special immunizations clinic at USAMRIID for laboratory workers at risk for exposure to *F tularensis*. This vaccine is efficacious, as documented in a human challenge model; however, this protection is not 100%, particularly at high-dose aerosol challenges.[56,106] In addition, an epidemiological study showed that the incidence of typhoidal tularemia in laboratory workers decreased significantly after the introduction of vaccination with LVS.[148] The primary disadvantages are the potential hazards associated with a live vaccine (such as severe infection in immunocompromised individuals) and the lack of effectiveness against high-dose respiratory challenge. For these reasons, there is much interest in the development of a subunit *F tularensis* vaccine.[11,12,149] Promising vaccine candidates are being explored.[12,149,150]

ISSUES FOR LABORATORY WORKERS

Tularemia is considered a significant hazard for laboratory workers.[114] All experiments that involve using *F tularensis* subspecies *tularensis* and fully virulent *F tularensis* subspecies *holarctica* strains should be conducted under biosafety level 3 conditions. Additionally, vaccination of at-risk personnel may diminish clinical manifestations of laboratory-acquired infections. A retrospective review of tularemia cases at USAMRIID was conducted. This study documented that typhoidal tularemia incidence dropped substantially after the LVS was offered to at-risk laboratory workers. Incidence rates decreased from 5.70 to 0.27 cases per 1,000 at-risk employee-years.[148] The occurrence of ulceroglandular tularemia did not decline significantly (from 0.76 to 0.54 cases per 1,000 at-risk employee-years), but milder symptoms were observed in the recipients of the LVS vaccine.[148] Another review of occupational exposures at USAMRIID suggested that the incidence of tularemia (15 cases/year) did not decrease with the introduction of biosafety cabinets, but did decline after LVS vaccination was introduced.[151]

USE OF TULAREMIA AS A BIOLOGICAL WEAPON

F tularensis could be used as a biological weapon in many scenarios, causing varying degrees of casualties. The most dangerous scenario involves an aerosol release with large numbers of persons exposed. Additional complications would result if an antibiotic-resistant strain—as is claimed to have been developed in the former Soviet Union—was used.[2]

Researchers have estimated that a large-scale aerosol release of 50 kg over a large metropolitan area could cause 250,000 incapacitating casualties.[29] Most of those affected could present with a nonspecific febrile illness 3 to 5 days after exposure (range: 1–14 days, depending on the inoculum of exposure), and would subsequently develop pulmonary symptoms consistent with pneumonic tularemia.[1] However, because of the aforementioned difficulties in tularemia diagnosis and the nonspecific clinical presentation, the determination of *F tularensis* as the causative agent may be delayed. The initial presentation of cases may be difficult to distinguish from a natural influenza outbreak or other respiratory pathogens.[1]

F tularensis may also be confused with another biological weapon. Epidemiological clues to distinguish tularemia from plague or anthrax are the clinical course of disease (slower with tularemia), case fatality rate (higher with plague[152] or anthrax[153]), and possibly the pattern of pulmonary manifestations observed on chest radiograph, such as the large pleural effusions and mediastinal widening characteristic of inhalational anthrax.[154] Pulmonary tularemia may be difficult to distinguish from Q fever, another potential biological weapon agent.

SUMMARY

F tularensis constitutes a substantial threat as a biological weapon. The variety of clinical manifestations of *F tularensis* infection and the benefits of early antibiotic intervention necessitate a high degree of suspicion from healthcare providers. Familiarization with the variety of epidemiological and clinical manifestations of this disease, along with available diagnostic tests and countermeasures allow healthcare professionals to minimize the impact of its use. Although the current LVS vaccine provides some protection against clinical disease associated with *F tularensis*, much interest remains in the development of a more effective vaccine. Further research will likely continue to elucidate the pathogenesis of this organism and yield improved preventive, diagnostic, and therapeutic options.

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Chapter 12

Q FEVER

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SUMMARY

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INTRODUCTION AND HISTORICAL PERSPECTIVES

Q fever is a zoonotic infection with almost worldwide distribution, characterized by sudden fever, headache, and atypical pneumonia, and which, in some cases, results in chronic disease. The causative agent of Q fever is the gram-negative bacterium *Coxiella burnetii***.** In two separate but concurrent instances, a new disease and the causative agent were discovered on different continents. An illness of unknown etiology struck slaughterhouse workers in Queensland, Australia, in 1933.[1,2] Symptoms included fever, headache, and malaise, but serological tests for all suspected infectious agents were negative. This led to the designation as query, or "Q," fever. Attempts to culture the agent on bacterial medium proved fruitless; however, scientists were able to demonstrate the transmissibility of the agent by inoculating guinea pigs with blood and urine from infected patients. It seemed likely that the causative agent was viral.

Around the same time in the United States, scientists in Montana were investigating a filter-passing agent isolated from ticks collected in the Nine Mile Creek area near Missoula, Montana. Initially looking for the agent of Rocky Mountain Spotted Fever, they found something new that did not cross-react with sera from patients infected with *Rickettsia rickettsii:* the causative agent of Rocky Mountain Spotted Fever, or any other known rickettsial agents.[3,4] When placed on guinea pigs, the ticks infected with this unknown agent caused febrile illness, which was transmissible to other guinea pigs by injection of blood from the infected animals. Examination of the inflammatory cells revealed that the agent was rickettsia-like.[4] Although the researchers in Montana were also unable to culture their agent on traditional bacterial media, they discovered that they were able to culture it in chicken embryos.[5] This discovery was a significant breakthrough not only for studying the Nine Mile agent, but also for the study of all rickettsial organisms. The scientists in Montana now had a Rickettsia-like microorganism that they had demonstrated to be infectious, but whether it caused disease in humans remained unknown until a researcher from the National Institutes of Health visited the public health laboratory in Hamilton, Montana, and became ill after working with the isolate from Nine Mile Creek. When blood from the researcher was injected into guinea pigs, the same febrile illness that resulted from the infected ticks was produced. After considerable effort by researchers in Montana and Washington, DC, a correlation was made between the agent found in the Nine Mile ticks and the disease that manifested in the visiting scientist. Serum from the visiting scientist was found to protect against infection by Nine Mile ticks in guinea pigs.[2]

The similarities between Q fever and the disease caused by the filter-passing agent found in the ticks from Nine Mile did not go unnoticed. In 1938, Dr Frank Macfarlane Burnet, who, along with Doctors Edward Holbrook Derrick and Mavis Freeman, published on Q fever in Australia, sent mouse spleens infected with Q fever agent to the National Institutes of Health in Washington, DC. The spleens were used to pass the infection to guinea pigs; the guinea pigs infected with Q fever were subsequently immune to the Nine Mile agent, but not the agents of Rocky Mountain Spotted Fever.[2] After issues arose with its source, Burnet's Q fever sample was lost before any further comparisons could be made; however, the similarities between the infections and immunity observed against the Nine Mile in guinea pigs that had recovered from Q fever infection indicated these two agents were one and the same.

Due to the similarities between the Q fever agent and members of the *Rickettsia* genus, it was originally classified as *Rickettsia diaporica*,[6] and then later *Rickettsia burnetii*,[7] in honor of Dr Burnet. In 1948, *R burnetii* was reclassified to its own genus named in honor of Dr Cox and became known as *Coxiella burnetii*.[8] Q fever's prevalence in the world was soon discovered; to date, it has been found in every country except New Zealand.

MILITARY RELEVANCE

Fevers of unknown origin and atypical pneumonia have plagued troops throughout history. Even as late as World War II (WWII), it was common for infectious disease to sideline significant numbers of troops and impact the outcome of battles. Because Q fever was identified just prior to WWII, it was during this war that the impact of the disease was first noted, though likely not understood to the full extent because of the nonspecific nature of the symptoms. While Q fever does not have a high mortality rate, it has the potential to debilitate large numbers of troops for extended periods of time.

World War II

Service members from both the Allied and the Axis forces sustained Q fever outbreaks during WWII. German troops were plagued with outbreaks of an atypical

pneumonia they called "balkengrippe" in northern Yugoslavia, Serbia, Bulgaria, Italy, Crimea, Greece, Ukraine, and Corsica in 1941.[9] The outbreaks were not well contained and a single consulting physician for the German army saw over 1,000 cases of balkengrippe. Although the mortality rate of Q fever is not high, the impact of these outbreaks was not insignificant; the minimum absence from duty for infected individuals was more than 6 weeks. Similar outbreaks occurred in 1942, 1943, and 1944, including a very severe outbreak in Swiss troops during which half of the soldiers in two battalion subunits developed pneumonia. As the balance of power shifted and Germany was pushed out of its previously conquered territories, the Allied troops now occupying these areas began to experience outbreaks of the same atypical pneumonia that had been sidelining German troops in previous years. From February to April 1945, there were 511 cases of "primary atypical pneumonia" admitted to British and New Zealand military hospitals near Naples, Italy. From winter 1944 to spring 1945, there were nine significant outbreaks of atypical pneumonia reported in Allied troops in Greece, Italy, and Corsica.[9] Although most of the outbreaks of atypical pneumonia were never confirmed to be Q fever during the acute stage of infection, serological analysis of select individuals up to 2 years after infection revealed high antibody titers against *C burnetii*.[9]

In the years following WWII, there were several small outbreaks of Q fever in military personnel. Between 1951 and 1958, there were three outbreaks of Q fever in Libya, Algeria, and the Isle of Man that impacted US, French, and British units, respectively. There were no further reports until an outbreak among British soldiers in Cyprus in 1974 that affected 78 soldiers.[10,11]

Gulf War

There were very few confirmed cases of Q fever in American service members during the Persian Gulf War. Only one case of acute illness was attributed to Q fever during this campaign: a severe case of meningoencephalitis associated with acute Q fever in a soldier following deployment.[12,13] Three additional soldiers within the same battalion as the afflicted soldier seroconverted during the same period, as evidenced by subsequent testing.[14] This small number of confirmed cases should not be taken as an indication that Q fever did not affect other military personnel or that it was absent in the environment. The nondescript nature of the symptoms associated with Q fever generally lead to gross under estimations of the disease.

Operation Enduring Freedom and Operation Iraqi Freedom

The impact of Q fever on military personnel in Iraq was realized starting in 2003.[15] To date, there have been over 150 cases of Q fever in US military personnel in Iraq and Afghanistan.[16–23] An evaluation of pre- and post-deployment sera of US military personnel hospitalized with symptoms consistent with acute Q fever between April 2003 and December 2004 revealed a seroconversion rate of 10%.[24] In addition to pneumonia, the infected personnel also presented with hepatitis, high fever, cholecytitis, and meningoencephalitis. In May of 2010, the Centers for Disease Control and Prevention issued an official health advisory detailing the potential for Q fever in travelers returning from Iraq.[25]

INFECTIOUS AGENT

C burnetii is classified phylogenetically within the gamma subdivision of proteobacterium in the *Legionellales* order, with several unique properties.[26] It is a pleomorphic coccobacillus with a gram-negative cell wall and replicates within phagolysosome-like parasitophorous vacuoles (PVs) of eukaryotic cells. Replication depends on trafficking to a PV with low pH; this "biochemical stratagem" as an acidophile[27] involves developmental cycle forms with metabolically active large cell variants and metabolically quiescent small cell variants.[28–30] The type isolate, Nine Mile (RSA493), was found to contain few highly degraded genes but a large accumulation of point mutations, leading to the hypothesis that *C burnetii* is in an early stage of reductive evolution.[31] This is in contrast to other obligate intracellular pathogens, like *Rickettsia* and *Chlamydia*, that appear to have undergone massive genome fragmentation and reduction since their separation from free-living organisms. *Coxiella* genomes are composed of one chromosome and one large plasmid or plasmid-related sequences integrated into the chromosome.[32–34] Sequencing genomes of four isolates identified approximately 2,150 to 2,300 open reading frames per genome and a high degree of homology among phylogenetically distinct isolate groups, with most major genetic variation resulting from transposon-mediated rearrangements.[35]

C burnetii undergoes a phase variation population shift where virulent phase I converts to avirulent phase II upon serial passage in a nonimmunologically

competent host.[36] Phase I organisms have "smooth" lipopolysaccharides (LPS) with complete O antigen, while phase II have a "rough" LPS, missing, at a minimum, O-antigen sugars. Two clonal isolates of the Nine-Mile-type strain—phase I, RSA493 (clone 7) and phase II, RSA439 (clone 4)—have been used for comparative analysis to demonstrate the requirement for O-antigen expression for virulence. The phase II isolate has a well-defined genetic deletion (~ 20 Kbp),[37] which encodes O-antigen LPS biosynthesis and will not revert to wild-type. This clone 4 has been exempted from Centers for Disease Control and Prevention's select agent regulation and requires only biosafety level 2 biocontainment.

C burnetii are primarily found in cells of the reticuloendothelial lineage, especially monocytes, macrophages, and polymorphonuclear cells during infection. Intracellular interactions have been modeled in vitro in a variety of continuous and primary cell lines, including L929 mouse fibroblasts, Vero (African green monkey kidney), J774 mouse macrophages, THP-1 human monocytes/macrophages, primary human peripheral blood mononuclear cells, and mouse bone-marrow-derived macrophages. The following models of the essential stages of uptake and survival have emerged from these studies.[38] *C burnetii* is taken up via complement receptor 3 and alpha V beta 3 integrin-mediated mechanisms into human macrophages.[39] The adhesins recognized by the host receptors are uncharacterized, but complement receptor 3 uptake appears dependent on loss of LPS O antigen.[39] An actin-dependent, endocytic mechanism of uptake leads to trafficking through an early endosome, progressing to a late endosome/phagolysosome PV.[40] The trafficking appears slightly delayed compared to latex bead uptake[41] and membrane markers for Rab5, Rab7, Rab24, LC3, lysosomal-associated membrane protein (LAMP)-1, LAMP-2, and LAMP-3, and flotillin 1 and 2 progressively decorate the PV.[42–45] Connection to the autophagosome compartment appears essential to support a productive replication compartment, and PV membrane development to a spacious vacuole requires access to continual cholesterol biosynthesis.[46] The PV biogenesis process requires de novo synthesis of *C burnetii* proteins, suggesting actively expressed bacterial factors in the modulation of host processes.[44] Infection of activated macrophages appears to favor pro-survival stimulation through Akt and extracellular signal-regulated kinases (Erks) 1 and 2 and restriction of proapoptotic events in a caspase-dependent manner.[47] Alternatively, the interaction with pro- and antiapoptotic host factors appears to be additionally complicated based on the observations that infected monocytic THP1 cells undergo a caspase-independent apoptosis soon after infection that may be mediated, in part, by tumor necrosis factor and require protein synthesis by *C burnetii*.[48,49] Infection of monocytes or macrophages does not result in subsequent activation, and several pathogen-associated common molecules appear modified to avoid serving as agonists for toll-like receptor recognition, including the lipid A of LPS that contains a tetraacylated structure with antagonistic activity for toll-like receptor 4.[50] When taken up by polymorphonuclear leukocytes, an incompletely defined, secreted acid phosphatase prevents release of reactive oxygen intermediates through the nicotinamide adenine dinucleotide phosphate-oxidase pathway.[51,52] This avoidance of activating macrophages appears to be a key pathogenic strategy, and hypersensitivity to oxidative stress suggests avoidance of reactive oxygen intermediates as well as detoxification as evolutionary pressures.[53,54] Through reductive evolution, several virulent isolates have lost expression of a functional catalase or secreted superoxide dismutase without a loss of virulence, and the organism requires very low iron levels, in part, as a strategy to avoid Fenton chemistry.[53,55]

The model of pathogen–host interaction suggests that *C burnetii* actively remodels the host cell to establish a productive intracellular niche, and two secretion systems are likely key to the effector molecule release that mediates this remodeling. Virulent isolates with acute disease-causing potential appear to encode a functional type IV pilus-structured, type II secretion system to release several enzymes, including acid phosphatase, phospholipases D and A1, copper/zinc superoxide dismutase, chitin, and a family of enhanced entry proteins.[55] *C burnetii* also encode and express a type IVB secretion system with striking similarity to the defective in organelle trafficking/intracellular multiplication (dot/icm) system (type IV secretion system [T4SS]) of *L pneumophila*.[31,56] It encodes 23 of the 26 dot/icm proteins, lacking homologs of the chaperone IcmR and the inner membrane proteins DotJ and DotV.[31,56,57] *C burnetii* dotB, icmS, icmW, and icmT are able to complement these mutations in *Legionella*,[58,59] while icmX, icmQ, dotM, dotL, dotN, and dotO do not complement.[59] These results suggest strong functional similarities as well as unique properties associated with each system. The *Legionella* dot/icm system has identified over 300 substrate effector proteins using a variety of approaches, and most of these effectors have been shown to subvert some step in the host cell process connected with replication in its unique niche.[60] Interestingly, although not surprising given the PV of *Legionella* and *Coxiella* are so dramatically distinct, *C burnetii* encodes relatively few homologs of the *Legionella* effectors. This suggests that although

C burnetii has maintained a functional dot/icm-related T4SS, the effectors, which are substrates for this secretion system, are almost entirely unique to *C burnetii*.

Pursuit of molecular pathogenesis studies for *C burnetii* has dramatically advanced with the development of the extracellular growth media acidified citrate cysteine media under microaerophilic conditions and the advent of genetic tools to randomly and site-specifically mutagenize and rescue mutant phenotypes using complementation methods.[61–66] Using these techniques, the isolation of T4SS mutants verified that, like *Legionella*, the dot/icm system of *Coxiella* is essential for intracellular replication but not growth in artificial media.[67,68] The identification of specific effectors released via T4SS has begun to determine which effectors are essential for this replication and potentially many additional *Coxiella*-unique virulence properties. Among potential type IV secreted effector molecules are a diverse family of pathotype-specific ankyrin repeat-containing proteins.[57,69] Ankyrin repeat domains (Anks) are commonly found in eukaryotic systems to mediate protein-to-protein and protein-to-DNA interactions and may be involved in host modulation events. Like *Legionella*, some redundancy appears to exist among *Coxiella* secretion substrates, as three substrates, ankyrin repeat-containing protein G (AnkG), *C burnetii* anti-apoptotic effector A protein (CaeA), and *C burnetii* anti-apoptotic effector B protein (CaeB), appear to act to promote host cell viability by modulating apoptosis. Recent studies suggest that AnkG binds the proapoptotic protein p32 to inhibit apoptosis.[70] Either redundantly or in support of AnkG, CaeB inhibits apoptosis through a mitochondrial pathway, whereas the nuclear effector CaeA was also noted to block apoptosis.[71] A variety of essential host manipulations via T4SS substrate/effectors released into the host cytoplasm will likely be identified to elucidate the intimate relationship between host and pathogen in this exquisitely adapted agent.

Pathogenesis

The pathogenesis of Q fever in humans is not well studied, and knowledge of acute *C burnetii* infection has been elucidated primarily in animal models. Upon inoculation, *C burnetii* are engulfed by resident macrophages and transported systemically. Alveolar macrophages have been identified as the resident cells that are primarily infected upon aerosol infection.[38,72,73] *C burnetii* grows and replicates within these macrophages and then bursts the cell, resulting in release and the subsequent infection of other phagocytic cells. In mice and guinea pigs, the spleen and liver are the most heavily burdened organs, and it is assumed that this is the same in human infection.[74] Chronic Q fever is far more complex. In chronic infection, reactivation of the microorganisms is possible years after the initial infection. Studies in guinea pigs and mice have demonstrated that these animals remain infected throughout their lives, but growth is uncontrolled during parturition and other periods of immunosuppression. Immunosuppressed animals have also been used to model chronic infection,[75] as have mice that are altered genetically to over produce the cytokine interleukin 10.[76] The early prediction that phylogenetic groups are uniquely virulent to cause either acute or chronic disease in humans is supported by studies that demonstrate in acute disease animal models that pathotypes are distinct in their ability to cause acute inflammatory disease.[77] Yet, there remains much to learn about chronic infection, including where the microorganisms persist during periods of latency and if the microorganisms are ever cleared from the body.

Epidemiology

Q fever is a worldwide zoonotic infection found in every country, with the exception of New Zealand. *C burnetii* is able to infect a wide range of species, but symptomatic infection is only found in humans. Occupational exposure is the primary source of human infection, with the vast majority of cases occurring in abattoir (slaughter house) workers, farmers/ranchers, and veterinarians. Although Q fever is primarily a problem in rural areas with domestic animals such as cattle, sheep, and goats as the primary sources, domestic pets can spread infection in urban areas, though at a much lower rate.[78,79] Infected animals shed *C burnetii* in urine, feces, milk, and, in highest concentration, in the placenta and other materials that are released during birth. *C burnetii* is incredibly stable in the environment and can persist as dried infectious particles for months or even years, perpetuating the infectious cycle.[80,81] This can also result in infections in individuals indirectly associated with the infected animals, such as those living in the area, because infectious particles can be carried by the wind. Although infectious aerosols are the most common source of infection in humans, bites by infected ticks and consumption of contaminated milk are also associated with infection[82] (though it is possible that long-term consumption of contaminated milk may result in seroconversion without causing disease).

C burnetii is one of the most infectious organisms known, with an infectious dose of fewer than 10 microorganisms, and possibly as low as 1 microorganism.[83,84] Routes of infection include aerosol, ingestion, and, rarely, human to human. The route of infection impacts

the manifestation of the disease; ingestion generally results in granulomatous hepatitis, while pneumonia is more common with aerosol transmission.[85] *C burnetii* is ubiquitous in the environment, yet there has been little work done to estimate its seroprevalence around the world. In the United States, the estimated seroprevalence is around 3% but the number of reported cases is very low, indicating that the majority of cases do not produce clinical disease.[19] Extensive studies were completed in Nova Scotia before 1990 that revealed a 14% seropositivity rate, but significantly fewer reported cases.[86,87]

Between 2007 and 2010, the Netherlands experienced one of the most significant Q fever outbreaks known. Approximately 4,000 cases of human Q fever were reported during this 3-year period, an exponential increase from the 1 to 32 cases per year that the country experienced in the years prior to the outbreak.[88,89] The outbreak resulted in the culling of millions of domestic animals, which was a devastating loss to the farming industry. This outbreak highlights the potential for large-scale Q fever outbreaks even in highly developed countries.

DISEASE

Humans (Q Fever)

Q fever is a zoonotic infection that produces symptomatic infection only in humans. Even in humans, only approximately 50% of infected individuals develop clinical disease, and the mortality rate is less than 1%.[1] Typically, Q fever presents as an acute, self-limited, systemic infection after an incubation period ranging from a few days to several weeks; there is evidence that disease severity is directly related to the infectious dose.[83,90,91] Symptoms, although acute, are typically nonspecific and may include fever, chills, severe headache warranting lumbar puncture,[19] fatigue, pneumonia, myalgia, nausea, vomiting, diarrhea, chest pain, weight loss, and abdominal pain. Of infected individuals, 30% to 50% develop pneumonia, and it is not uncommon for patients to develop hepatitis. Less common clinical manifestations include acalculous cholecystitis, acute respiratory distress syndrome, gastroenteritis, myelitis, orchitis, epididymitis, pericarditis and myocarditis, and rhabdomyolysis, among others.[12,17–20] In many cases, patients clear the infection without antibiotic intervention.

In addition to acute infection, Q fever is also capable of inducing chronic infection. Chronic Q fever has the potential to manifest in excess of 20 years after the initial exposure and can persist for 6 or more months at a time. In its chronic form, Q fever is especially dangerous in patients with heart disease, as endocarditis is known to develop in conjunction with chronic disease. Immunocompromised patients, such as those undergoing immunosuppressive therapy, suffering from diseases such as AIDS, or receiving antirejection therapy after organ transplant are particularly at risk for developing chronic Q fever.[92,93] The most common disease associated with chronic Q fever is endocarditis of the aortic and mitral valves, though it is not uncommon to see chronic hepatitis.[94–97] A preexisting heart disease is a major risk factor for chronic Q fever. It is estimated that of those individuals that develop Q fever endocarditis, close to 90% have preexisting valvular disease[96]; as many as one third of all chronic Q fever patients with cardiac valve abnormalities go on to develop Q fever endocarditis.[97] In patients with chronic Q fever, the immune responses necessary to fight the infection, such as T-cell responses, are not present. Patients have been observed to produce increased immunosuppressive cytokines, including tumor necrosis factor[98] and interleukin 10.[76,99] Immune response suppression is responsible for the persistence of the microorganism.

Animals (Coxiellosis)

As a zoonosis, infection with *C burnetii* is termed "coxiellosis" and infects both wild and domestic animals. Q fever is especially common in domestic ruminants such as cattle, sheep, and goats. Unlike infection in humans, coxiellosis does not produce overt symptoms, and pathological changes are generally limited to the genital tract, manifesting as spontaneous abortion and fertility issues.[100] Sheep appear to have only transient infection while the infection persists in other mammals.[101] Infection reactivates in female mammals during pregnancy. As with infection in humans, animals are infected by aerosol transmission, tick bites, and milk (nursing). A 2005 study demonstrated that over 90% of dairy cows in the northeastern United States are infected with *C burnetii*,[102] but pasteurization prevents human infection via consumption of contaminated milk. Although exposure via contaminated milk can be prevented by simply pasteurizing milk, infection by the aerosol route (ie, inhalation of dried infectious particles shed by infected animals) cannot be prevented. Without obvious symptoms, it is difficult to identify infected animals and therefore next to impossible to eradicate *C burnetii* from domestic animals.

DIAGNOSIS

Q fever cannot be diagnosed based on clinical symptoms because of the nonspecific presentation of symptoms; instead, diagnosis is made based on the combination of clinical signs and serological testing. History of exposure to animals or time spent on farms bolsters what is usually a presumptive diagnosis. Humoral responses are more consistently activated during Q fever infection than cellular responses and, as such, serological testing is considered the more reliable immunoassay for Q fever. The most commonly used assays are indirect immunofluorescence assay (IFA), complement fixation, microagglutination, and enzyme-linked immunofluorescence assay (ELISA).[19,103]

The gold standard for Q fever serological diagnosis is the IFA because it is highly sensitive and very specific, and does not require purified antigen.[103,104] This method is very convenient for laboratories with limited space for equipment, but it is not feasible for testing large numbers of samples. Complement fixation is one of the most specific assays for diagnosing Q fever, but it lacks sensitivity and cannot detect specific antibody early in the course of an infection.[105] Microagglutination is sensitive, but its use is hindered by the requirement for large amounts of antigen.[103,106] ELISA rivals IFA in specificity and sensitivity, and is a platform conducive for analyzing large numbers of samples. Unlike IFA, however, ELISA requires highly purified antigen to achieve sensitivity and specificity.[103,104,107]

Phase variation plays an important role in serodiagnosis of Q fever. Acute and chronic Q fever produce characteristic, yet distinct, antibody profiles.[108–111] During acute infection, antibody to the phase II, nonpathogenic organism is detectable before antibody to the phase I, pathogenic organism. Phase II antibody titers peak much higher than phase I antibody and remain elevated for years after infection, while phase I antibody titers wane shortly after infection. Individuals with chronic infection have an antibody profile exactly opposite the profile of individuals with acute infection. Chronic infection produces and sustains high titer phase I antibody, but much lower phase II antibody titers.

Although it is possible to diagnose Q fever based on bacterial culture, this method is not widely used. *C burnetii* is highly infectious and must be grown under biosafety level 3 conditions, a requirement that is not feasible at most medical treatment facilities. Culturing *C burnetii* from patient samples can be done by using the sample to infect research animals such as mice, or by infecting a monolayer of cells and subsequently staining and visualizing the cocci. These methods are very time consuming and are not conducive for large numbers of samples.[103] The advent of the use of acidified citrate cysteine media[65] for axenic culture of *C burnetii* will likely make culturing patient samples easier, but it seems unlikely that this will become the primary method for diagnosing Q fever. Detection by quantitative polymerase chain reaction is also a viable method and can be used to detect the bacteria in the infected individual much earlier than methods that rely on the production of antibody.[112,113] Most of the information on the use of PCR for diagnosis have come from animal studies, but there has also been success detecting *C burnetii* in the buffy coat of citrated or ethylenediaminetetraacetic acid (EDTA)-treated blood.[103] In the spring of 2011, the US Food and Drug Administration cleared the first test to diagnose Q fever in military personnel serving overseas. The nucleic acid amplification test by Idaho Technologies produces results within 4 hours.[114]

TREATMENT

Acute Q fever is easily treated. The treatment of choice is doxycycline, 100 mg twice daily for 14 days.[115] This treatment is not effective for chronic Q fever; instead, drug combinations such as doxycycline plus hydroxychloroquine are considered the most efficacious treatment in chronic cases. In cases with endocarditis, an 18-month regimen of 100 mg of doxycycline twice per day and 200 mg of chloroquine three times per day is effective.[117] When chloroquine is not an option for a particular patient, a combination of 100 mg doxycycline twice per day and 200 mg ofloxacin three times per day is recommended for a period of 3 years.[116] There is evidence of reactivation of disease when the drug regimens are shortened or not completed. Doxycycline is not bactericidal, but is an effective treatment for intracellular bacteria such as *C burnetii* and *Chlamydia*. The effectiveness of doxycycline is improved when used in combination with hydroxychloroquine and it is hypothesized that the hydroxychloroquine increases the pH of the phagolysosome, which would decrease the metabolic activity of *C burnetii*.[117]

VACCINE

The ability to create an efficacious vaccine has never been a problem in the field of Q fever research. The failure of most of these vaccines has been the inability to uncouple the protection and the accompanying adverse reactions. Within a few years of identifying *C burnetii*, researchers had successfully developed an effective vaccine.[118] The composition of the vaccine was crude and consisted of formalin-inactivated *C burnetii* extracted with ether from egg culture and was contaminated with 10% yolk sack. Knowledge of the phase variation that occurs in *C burnetii* came after this early vaccine was developed. It was later determined that vaccines consisting of phase I antigen are 100- to 300-fold more protective than those consisting of phase II antigen in guinea pigs.[119] Whole cell vaccines (WCVs) consisting of formalin inactivated phase I *C burnetii* have been studied extensively. There is no question that these vaccines are highly protective against Q fever, but the side effects that occur in a specific population of people has prevented their wide-spread use. Individuals that have been exposed to *C burnetii* prior to vaccination, such as those who have had Q fever or have spent significant time in close proximity to livestock, are very likely to have adverse reactions at the site of injection.[120,121] The reactions can range from mild redness and swelling to painful granulomas and sores. The same can be seen after multiple vaccinations. To circumvent the adverse reactions to the WCV, alternative antigen sources have been, and continue to be, investigated.

M-44

The Soviet Union developed a live attenuated oral vaccine using phase II organisms from the M-44 strain.[122,123] The vaccine was tested in Soviet volunteers with great success, but studies in guinea pigs revealed that the organisms persisted in the animals and led to lesions in the heart, spleen, and liver, suggesting either reactivation or low-level contamination with phase I organisms.[124]

Chloroform:Methanol Residue

Another attempt to prevent the side effects of vaccination in the previously exposed individuals was to vaccinate with an extraction from phase I organisms, rather than the WCV. Scientists at the United States Army Medical Research Institute of Infectious Diseases vaccinated with the residue from chloroform and methanol extracted from phase I organisms that had been formalin inactivated. They have demonstrated that the vaccine is protective in animals, nontoxic, and immunogenic in humans.[125,126] However, at high doses the residue is reactive, though to a lesser extent than whole cell vaccine. Studies using a low-dose prime-boost scheme have shown success in animals and induce protection without reaction.[127]

Q-Vax

Q-Vax (short for Q fever vaccine) is a WCV that is licensed for use in Australia from CSL Limited, a company based in Victoria, Australia; it is the most widely studied Q fever vaccine.[128] Studies have demonstrated that this vaccine is 100% effective for more than 5 years in individuals who are considered extremely at risk due to their occupation.[128] Q-Vax, however, is hampered by the need for pretesting to determine prior immunity. It is currently not licensed for use in the United States.

SUMMARY

Q fever is a worldwide zoonotic disease with one of the most highly infectious causative agents known, *C burnetii.* Q fever significantly impacted wartime efforts as recently as the past few years, and as evidenced by the recent outbreak in the Netherlands, can produce prolific disease even in areas with sufficient preparation. The ubiquitous nature of *C burnetii* in the environment indicates that Q fever will be a concern for years to come; not only does it have the potential to produce disease in humans, it can also result in devastating losses to domestic animals. Extremely resistant to environmental stresses, *C burnetii* can persist for long periods of time in the environment.

Q fever is easily treated by antibiotics of the tetracycline class, though diagnosis can be difficult based on the nonspecific symptoms observed in Q fever patients. Physicians must rely on a combination of clinical presentation and serological testing to diagnose Q fever. Although the vaccine used in Australia is not available for use in the United States, efforts continue to find a safe and efficacious alternative.

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Chapter 13

MULTIDRUG-RESISTANT BACTERIAL INFECTIONS AS A THREAT TO THE US MILITARY HEALTH SYSTEM: *ACINETOBACTER* INFECTIONS AS A CASE STUDY

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INTRODUCTION

EPIDEMIOLOGICAL CONSULTATION
- **Mission**
- **First Reservoirs in the Combat Zone**
- **Nosocomial Infection as the Dominant Problem**

MOLECULAR ANALYSIS
- **Strain Collection and Sampling**
- **Genetic Analysis, Characterization, and Identification of the Source**
- **Antibiotic Resistance**

MILITARY HEALTH SYSTEM RESPONSE TO *ACINETOBACTER* AND OTHER "ESKAPE" PATHOGENS
- **The Way Forward**

SUMMARY

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INTRODUCTION

Although not new to the civilian critical care community, infections with multidrug-resistant (MDR) *Acinetobacter* species were rare in the US Military Health System (MHS) before the wars in Afghanistan and Iraq. In fact, the marked increase in such infections immediately following these conflicts led some to postulate that MDR *Acinetobacter* species may be a result of human engineering with malicious intent, leading some news reports to refer to it as "Iraqibacter." However, there is no evidence supporting this contention. *Acinetobacter* species are environmentally hardy and difficult to eradicate from inanimate healthcare surfaces, but their relatively low virulence makes them poor candidates for weaponizing. In fact, even in the most severely war-wounded patients, *Acinetobacter* species infections are rarely fatal.[1,2] Nonetheless, MDR *Acinetobacter* species pose an equally concerning risk to global public health as bacteria engineered for weapons use.

Infection has always been a complication of war trauma. Treatment of traumatic wound infections has evolved over time, as a belief in "laudable pus" yielded to surgical debridement, and the emergence of penicillin in 1942 ushered in a period when recovery from serious infections became possible, if not expected. However, the 21st century has witnessed the expansion of bacteria that are resistant to multiple antibiotics, and a dearth in new drug development has resulted in infections from bacteria that are resistant to virtually all available antibiotics. These MDR bacteria have become well recognized in hospitals around the world and are especially problematic in locations where antibiotic use is frequent, such as in intensive-care units and long-term acute care facilities. During Operation Enduring Freedom (OEF) and Operation Iraqi Freedom (OIF), forward-deployed US medical facilities often provided acute care to service members who were rapidly medically evacuated, as well as to local national patients who required sustained care. Antibiotic use following traumatic injury to prevent or treat infection was the standard of care, and thus the stage was set for the selection of MDR bacteria, which subsequently spread through the evacuation chain. Although several different species of MDR bacteria emerged to complicate war trauma care, *Acinetobacter calcoaceticus baumannii* (ACB) complex first heralded the problem, which led to the investigations discussed in this chapter.

Bacteria of the genus *Acinetobacter* are glucose nonfermentative, nonfastidious, catalase-positive, oxidase-negative, strictly aerobic, gram-negative, coccobacilli (or pleomorphic) and commonly occur in diploid formation or in chains of variable length. However, different genospecies cannot be easily identified using traditional methods. Members of the genus have been classified in various ways; therefore it is difficult to understand the true status of the epidemiology and clinical importance of these organisms. Since 1986, the taxonomy of the genus *Acinetobacter* has undergone extensive revision. The original single species named *Acinetobacter calcoaceticus* has been abandoned, and at least 32 genospecies have now been proposed, 17 of which have been correlated with species' names. Identifying the members of the genus *Acinetobacter* to the species level by traditional methods is problematic. *Acinetobacter baumannii* (genospecies 2), *Acinetobacter pittii* (formerly known as *Acinetobacter* genospecies 3), and *Acinetobacter nosocomialis* (formerly known as *Acinetobacter* genospecies 13TU) are genetically and phenotypically similar to *A calcoaceticus* (*Acinetobacter* genospecies 1) and hence are grouped in the so-called ACB complex. Molecular methods are needed to identify members of the complex to the species level because each member has a distinct antimicrobial susceptibility profile and shows different clinical characteristics. In this chapter, we use the term *Acinetobacter* or ACB interchangeably and to indicate the overall phenotype that includes the four most clinically relevant species mentioned above. These have gained notoriety for a predilection to cause nosocomial infections and to develop resistance to multiple antibiotics.[3]

The importance of ACB has been recognized with its inclusion in the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, ACB, *Pseudomonas aeruginosa*, and *Enterobacter* species), a group of bacteria identified by the Infectious Diseases Society of America that risk becoming resistant to all available antibiotics.[4] ACB is predominantly associated with medical technology, as most infections occur in the setting of artificial ventilation (ventilator-associated pneumonia), intravenous access (line-associated bacteremia) or urinary catheterization (urinary tract infections).[5] Although spontaneous, invasive infection of immune-competent patients appears to be uncommon, infections associated with trauma have been reported. Following an earthquake in Turkey in 1999, a local hospital experienced an 18.6% rate of hospital-acquired infection (HAI; predominantly wound infections) with MDR ACB being the most commonly isolated pathogen.[6] In 2002, 62% of trauma victims following a bombing in Bali had ACB infections, and in 2004 MDR ACB was discovered in 18% of cultures among injured tsunami victims evacuated from Thailand to Germany.[7,8]

The presence or relative absence of ACB as a "militarily relevant" pathogen prior to 1970 is unknown. Among casualties of the Korean War, at least one blood culture was reported to grow *Achromobacter* species,[9] and during the Vietnam conflict, the predominant gram-negative pathogen recovered from a series of 30 Marines was in the *Mimeae-Herellea-Bacterium-Alcaligenes* group, which is postulated as being ACB. However, it is unclear if any of these bacteria would be classified as ACB using current taxonomy. Furthermore, the organism was not prevalent in other studies of combat wounds during either war.[10–14] The numbers of US casualties in Operations Just Cause (Panama, 1989–1990), Desert Storm/Shield (Iraq, 1990–1991) and Restore Hope (Somalia, 1992–1993) were relatively low, and ACB infection was not reported.[15]

US forces entered Afghanistan in 2001 in support of OEF and Iraq in 2003 in support of OIF, and military healthcare providers shortly thereafter began noting an increase in the number of patients infected with ACB. A collaborative report from military physicians and the Centers for Disease Control and Prevention was published in 2004, highlighting 102 injured service members whose blood cultures grew ACB. Most of these cases were reported from Landstuhl Regional Medical Center (LRMC) and Walter Reed Army Medical Center (WRAMC), with 32 OIF and 29 OEF bacteremic patients. The number of patients with ACB bloodstream infections in 2003 and 2004 exceeded those reported in previous years (one case during 2000–2002 at LRMC, and two cases during 2001–2002 at WRAMC).[16] A review of 211 trauma casualties evacuated from Iraq to the United States Naval Ship (USNS) *Comfort* during the first month of OIF) in 2003 revealed 44 cultures positive for *Acinetobacter* species, representing 33% of all isolates. Specifically, 36% of wound isolates and 41% of bloodstream isolates were of *Acinetobacter* species.[17] A review of MDR bacteria at WRAMC demonstrated a marked increase in the incidence of ACB infections, peaking in 2004 (Figure 13-1), and a study using multilocus polymerase chain reaction (PCR) and mass spectrometry to genotype isolates demonstrated that some of the strains belonged to an atypical and evolving group of isolates, distinct from those found at nonmilitary hospitals in the United States.[18,19] Additionally, molecular typing of isolates from one of the worst outbreaks of MDR ACB in the MHS occurred at WRAMC and revealed eight major clone types, 60% of which were related to the three International Clonal Complex (ICC) types, suggesting multiple independent origins.[20, 21]

The clinical impact of the ACB outbreak associated with OIF/OEF was acute, with lingering downstream effects. A retrospective study of 93 war-related trauma patients at WRAMC with ACB bacteremia determined

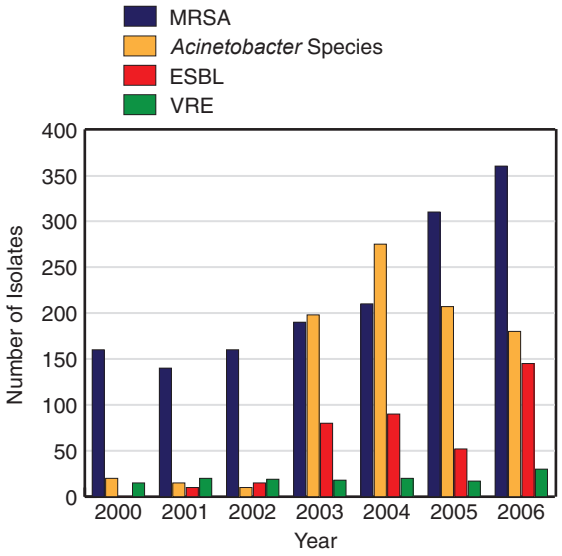


Figure 13-1. Annual incidence of drug-resistant organisms isolated from clinical specimens at Walter Reed Army Medical Center (2000–2006).
ESBL: extended-spectrum β-lactamase-producing organisms
MRSA: methicillin-resistant *Staphylococcus aureus*
VRE: vancomycin-resistant enterococci
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that there was a median of 1 day between admission and detection of infection. Of these isolates, 86% were resistant to multiple antibiotics. The 30-day mortality rate in this group of patients was only 2%, and those 2 patients died from massive pulmonary emboli believed to be unrelated to ACB bacteremia. The authors concluded that the absence of severe comorbidities (as measured by the Acute Physiology and Chronic Health Evaluation II score and Charlson comorbidity index), compared to cohorts of patients in other published reports of ACB bacteremia, contributed to the low mortality rate.[1]

Along with bacteremia, military providers began noting the development of ACB wound and burn infections, as well as osteomyelitis. Davis et al reported 23 patients with MDR ACB, 18 of whom had osteomyelitis, 2 with burn infections, and 3 with deep wound infections. Within a mean follow-up period of 9 months, all patients were cured of infection.[22] ACB was reported to be the most prevalent organism recovered from military burn patients injured during operations in Iraq and Afghanistan, with the percentage of ACB resistant to four classes of antimicrobial agents increasing from 17% in 2003 to 2005 to 49% in 2006 to 2008.[23,24] Trauma-related skin and soft-tissue infections were reported by Sebeny et al, who reported their findings

in eight patients with infection due to ACB.[25] Osteomyelitis was noted in several cohorts, often in association with orthopedic fixation devices.[26,27] ACB central nervous system infection associated with trauma was reported in several cases.[28,29]

In addition to causing infectious complications in traumatically injured patients, ACB developed resistance to multiple classes of antibiotics, posing treatment challenges to clinicians. At both WRAMC and Brooke Army Medical Center (BAMC), the prevalence of MDR ACB rose dramatically during the course of OIF/OEF. At BAMC, the percentage of MDR ACB rose from 4% to 55% between 2001 and 2008, while at WRAMC the percentage of ACB that was susceptible to imipenem dropped from 100% in 2002 to 61% in 2006.[19,30] Faced with the loss of traditional antibiotics, physicians turned to other agents, including tigecycline, minocycline, and colistimethate sodium.[31–33] The switch to second-line agents came at a cost, however, as the incidence of acute renal failure (defined as the first three criteria in the RIFLE acronym [risk, injury, failure, loss, and end-stage renal disease]) in 66 patients who received colistimethate sodium was 45%; and 21% of patients stopped therapy because of nephrotoxicity. Fortunately, renal function returned to normal once colistimethate sodium was stopped.

In summary, prior to OIF/OEF, ACB had been recognized as a pathogen among hospitalized patients, and scattered reports had noted an association with civilian trauma. However, its emergence as a pathogen associated with war trauma was unexpected, as was the breadth of its clinical presentation (including bacteremia, skin and soft-tissue infection, meningitis, and osteomyelitis). The organism's ability to develop resistance to multiple antibiotics complicated treatment decisions, leading to the use of more toxic agents. Healthcare on the modern battlefield now extends from the point of injury to US-based, Echelon V (Level V) facilities, separated by a transit time of only a few days. Traumatically wounded patients are cloistered in an intensive-care environment, supported by invasive medical devices (ventilators, chest tubes, urinary catheters and intravenous lines), and administered antibiotics that invariably select for resistant pathogens. In retrospect, the emergence of a nosocomial pathogen was perhaps inevitable, and ACB was well positioned to exploit the opportunity. Trauma-induced disruption of anatomic barriers to infection and antibiotic use appear to select for ACB, and thus the military healthcare system will likely face this challenge again in future conflicts.

EPIDEMIOLOGICAL CONSULTATION

Mission

Lieutenant General James B Peake, who was Surgeon General of the Army and Commander, US Army Medical Command, ordered an epidemiological consultation (EPICON) on August 27, 2004. The charge to the US Army Center for Health Promotion and Preventive Medicine (now the Army Public Health Command) included objectives that had typified military epidemiological investigations for decades and now addressed ACB. Descriptive and risk-factor analysis, identification of sources of infection, and recommendations for control, prevention, and future surveillance were all expected. However, the scope and complexity of the ACB problem prompted an approach that differed substantially from previous outbreak responses. A multicenter effort was organized that included the Walter Reed Army Institute of Research (WRAIR) and the Department of Defense Global Emerging Infections Surveillance and Response System (DoD-GEIS), in addition to four hospitals in the chain of medical evacuation from Southwest and Central Asia: the 31st Combat Support Hospital (31st CSH in Iraq, Level III), LRMC (Level IV), WRAMC (Level V), and BAMC (Level V). Twenty principal personnel, including four civilians, provided data or conducted local studies to support the investigation; these included clinicians, epidemiologists, infection control practitioners, microbiologists, an environmental scientist, and a statistical programmer. Numerous other individuals from various military organizations served as consultants or collaborators, including Navy and Air Force personnel, and advice from medical and laboratory contacts with ACB experience in other countries, particularly those in Southwest Asia, was also obtained.[34]

The three-way networking of public health, research, and clinical assets was not a new endeavor; neither was applying tools like genetic fingerprinting, web-based data collection, and multipoint conferencing. Nevertheless, bringing these together in response to the ACB problem seriously threatening hospitalized military beneficiaries set a new precedent for the MHS; just as the coordinated, international response to severe acute respiratory syndrome, on a broader scale, had set a new precedent for global public health the previous year.

Acinetobacter infections had been rare at LRMC and WRAMC; but cases began to emerge in 2002 during the first year of OEF in Afghanistan and their frequency accelerated immediately after OIF began in March 2003.

Since microbiology and clinical data within military hospitals were archived, confirmation of an outbreak at LRMC and WRAMC was rather straightforward. Even after adjusting for the number of admissions, intensive-care bed days, and the frequency of culture specimen collection, rates of all types of ACB infection exceeded historical counts at these hospitals. At LRMC, 36 bloodstream infections were observed in 2003 and 2004, compared to only 4 in the preceding 3 years. At LRMC and WRAMC combined, the average number of ACB isolates had increased from 1 per month during 2002 to 1 per day during 2003 and 2004.[34]

Among the aims of data analysis and interpretation by EPICON investigators were: (*a*) weighing the relative importance of wound contamination at the time of injury with that of nosocomial infection; (*b*) determining the role of Southwest or Central Asia as a geographic source, versus a primary role of intensive hospital care regardless of geographic location; and (*c*) distinguishing the importance of fomite versus person-to-person transmission as an underlying propagation mechanism. It was not assumed *a priori* that these were mutually exclusive dichotomies; and much was already well known about ACB as a species complex, such as the ability of the organisms to colonize humans in addition to the inanimate, or built environment of the hospital, and soil and water.[35] The newly accumulated evidence was mixed with respect to each of these aims when considered individually; however, taken together, the findings empirically supported a set of practical countermeasures and ongoing surveillance procedures.[34]

First Reservoirs in the Combat Zone

A baumannii strain distribution across international borders has been described, with evidence of drug resistance transfering between globally distant hospitals.[36,37] Evidence of drug-resistant *A baumannii* as a growing problem in Southwest Asia was revealed during the outbreak in US military hospitals, both through investigators' conversations with hospital infections experts in the region and through published reports.[38,39] Also, surveillance at the 31st Combat Support Hospital in Baghdad had shown that non-US patients (coalition and local national) were colonized with ACB at a proportion that was more than five-fold that of US patients. Among hospital staff, 15 pairs of hands had been screened in one series, and none of the specimens grew ACB. During a 2-month period after the EPICON was initiated, 102 screening specimens from the US field hospital in Baghdad were obtained from patients receiving care in the emergency treatment area or being admitted to intensive care. One (2%) of 64 US patients and 4 (11%) of 38 Iraqi patients were found to be colonized. Furthermore, the hospital ship providing Level III care in the Persian Gulf during the early weeks of OIF (USNS *Comfort*, discussed earlier) was receiving primarily Iraqi nationals when *Acinetobacter* isolates had become relatively common.[17,25]

Screening of 96 ambulatory patients evacuated from Iraq to Landstuhl from its usual catchment area revealed no ACB skin colonization. However, among 472 inpatients admitted to LRMC during the same period, 19 (3.9%) had skin cultures that were positive for ACB. Patients admitted to intensive care had a colonization prevalence of 10% (relative risk [RR] = 2.8 compared with regular ward admissions, $P < 0.0001$). Patients with ACB bloodstream infection were found to have the infection within 48 hours of arrival (over 50% on the day of admission), suggesting acquisition before admission to Level IV in the most seriously affected ACB patients.[34]

Analysis of locations in Iraq and Afghanistan from which patients were evacuated to Landstuhl over time revealed that the US hospital at Ibn Sina (Baghdad) contributed patients with the highest proportion colonized or infected with ACB, and that originating from field hospitals in Iraq presented a higher risk than from Afghanistan. When the results of colonization studies at three echelons (Baghdad, LRMC, and WRAMC, 2003 to 2004) were examined together, including stratification between admission and discharge screening at LRMC and WRAMC, the proportions of patients with positive ACB cultures showed a clear, progressive increase with each level of care.[34]

At least one culture of the inanimate hospital environment from each of seven field hospitals (five in Iraq, two in Kuwait) was positive for ACB. Specific sampling locations were documented for 37 isolates. All of these were subjected to 16S rDNA (ribosomal deoxyribonucleic acid) sequencing, and 34 also underwent pulsed field gel electrophoresis (PFGE). In addition, there were 170 isolates available from 145 individuals tested in 2003 while they were inpatients at Baghdad, the USNS *Comfort*, LRMC, or WRAMC. All of these underwent PFGE, and 164 of them underwent 16S rDNA sequencing. The results are in the Molecular Analysis section, but key to the issue of strain importation was the demonstration that 43 patients treated at 4 different military hospitals were infected with related strains from a single cluster group which, in turn, was genetically related to an isolate derived from environmental sampling of an operating room in the Baghdad field hospital. This group included both US and non-US patients, and both those who had and those who had not deployed to the Central Command area of operations. No cultures were positive for ACB organ-

isms when 31 archived soil samples from the general environment of Iraq and Kuwait were tested (collected March 2003 to December 2004 from various locations, not in the vicinity of hospitals).[21] A separate report, also published in 2007 but focusing on Canadian soldiers with *A baumannii* ventilator-associated pneumonia, similarly described genetic linkage between a field hospital environmental isolate (from a ventilator intake filter in Afghanistan) and isolates from four soldiers whose hospital treatment continued in Canada. Linkage to clinical specimens at Level IV (LRMC in these cases as well) was also found for three of the patients.[40]

Observations based on colonization and environmental studies of ACB may be confounded, biased, or diluted in significance when low-yield skin sampling sites are used for surveillance and colonization studies. This can also occur when reliance on routine cultures fails to distinguish *A baumannii*, the species most often causing opportunistic infection, from clinically less-significant species.[41,42] During the EPICON, investigators found poor agreement between different body sites (eg, axilla versus groin, hands, feet, or forehead) at LRMC and BAMC when skin cultures were observed for ACB growth; and most isolates from environmental sampling were ACB other than *A baumannii*. Thus, a separate focus on clinical specimens, and the subjecting of both clinical and environmental isolates to species identification and molecular typing, provided critical data for the investigation.

In addition to the EPICON, separate endeavors by Air Force personnel contributed data regarding ACB. In 2005, 83 environmental samples were taken from two C-141 aircraft used for aeromedical evacuation from Iraq to Germany, and from a deployed hospital of the Expeditionary Medical Dental Group (332nd EMDG, Balad Air Base, Iraq). The source locations included the walls, seats and floors from the front, middle, and back sections of the aircraft; the operating rooms (ORs) and wards of the hospital; and a variety of equipment (litters, litter straps, life pack monitor covers, and outer surface of endotracheal tubes). Also sampled were personnel working directly with patients while receiving, flying with, or transferring them. Three samples were taken from personnel working with patients from both the gloves and hands of caregivers. Finally, 16 of 58 patients who were transported during the observed flights were screened for ACB at LRMC. All of the environmental, equipment, personnel, and patient specimens were negative for ACB, except for one sample taken from a patient air warmer in the Balad hospital, which produced an imipenem-sensitive isolate. Of course, the possibility of colonization or contamination during transport of known, ACB-infected patients could not be ruled out.[34]

At the 332nd EMDG hospital, the surgical staff observed a significant reduction in ACB infections after implementing very aggressive infection control procedures, which indirectly supported the conclusion that the outbreak at higher echelons of care was likely preceded by nosocomial transmission in field hospitals. Countermeasures applied in Balad included strict enforcement of contact precautions as well as standardized intraoperative wound management, imposed conservative use of antimicrobials, and initiated special interventions (ie, plastic draping for all OR entrances, opening an additional OR for the most contaminated wounds, opening an additional ward to reduce patient crowding, greater use of heat and bleaching for linen cleaning, and thorough cleaning of ORs and wards, including weekly filter and duct cleaning in environmental control units).

Nosocomial Infection as the Dominant Problem

When the EPICON report was submitted in mid-2005, nosocomial transmission had been clearly documented in at least 39 *A baumannii* infection cases at WRAMC, and three quarters of these were cross infections in patients who were not evacuated from a deployed setting, including four civilians who died with bloodstream infection as an underlying cause. There had also been a fatal case at LRMC in a non-OEF/OIF patient: a 63-year-old woman who had been admitted to the ward with exacerbation of chronic obstructive lung disease, and who initially improved. Unfortunately the case went on to illustrate the serious impact ACB was having on persons completely unassociated with military operations. On hospital day 11, there was a sudden clinical deterioration, requiring the patient to be transferred to intensive care and mechanically ventilated. She expired the next day. Culture results supported the diagnosis of *A baumannii* pneumonia and bacteremia. During the patient's hospital stay, at least five patients colonized with *A baumannii* had been admitted to the same ward and one of them stayed in the same room, but PFGE analysis distinguished the colonized patient's strain from that of the deceased. However, an isolate completely matching that of the deceased was obtained from a patient staying in a different room on the same ward. This favored transmission by healthcare personnel over fomites in the room as an explanatory mechanism in this case.[34]

An analysis of nosocomial ACB transmission at WRAMC and BAMC revealed that the resulting infections primarily involved the respiratory tract, and that ACB acquisitions were primarily among civilian beneficiaries. Comparing 2004 with 2003 at LRMC and WRAMC combined, ACB wound specimens accounted for a diminishing fraction of positive

cultures, while bloodstream infections increased as a proportion of ACB infections. At LRMC the respiratory tract was considered a likely portal of entry for many of the nosocomial infections and, after excluding specimens taken from the skin and wounds, the respiratory tract was also the most common site of infection. The urinary tract was not a major site of infection, but infection there was more common at WRAMC than at LRMC.

The EPICON report concluded that

> While all stages of the military healthcare system can propagate or sustain the presence of *A baumannii* on patients, the initial source of the current outbreak appears to be the (Level III facilities) in Iraq and Afghanistan. Nosocomial transmission accounts not only for some of the infections at (higher echelons) but also for the initial infection of US troops who acquire the infection before or during strategic MEDEVAC [medical evacuation]. Patients with relatively long inpatient stays in these hospitals (especially non-US patients) represent a likely reservoir for transmission of the organism. Pre-hospital, primary wound infections in theater are not likely to have a significant role in transmission.[34]

With respect to environmental surface contamination versus colonized people, both were linked sufficiently to transmission to warrant both enhanced sanitation (room, equipment) and strict personal hygiene. Despite such measures, control of nosocomial transmission and of further progression toward drug resistance continued to prove extremely challenging for the MHS in the years following recognition of the *A baumannii* outbreak.[43] Nevertheless, it is highly likely that morbidity and mortality would have continued to increase without strict preventive interventions, as now promulgated in national guidelines.[44]

MOLECULAR ANALYSIS

Strain Collection and Sampling

Around April 2003, physicians in the MHS noticed a marked increase in the number of *Acinetobacter* infections within Level IV and Level V medical treatment facilities (MTFs). The formal investigation described in the previous section was launched the following year. A critical part of the EPICON was to, "identify the cause(s) or source(s) of infection." More than 200 clinical and environmental isolates were collected from 148 different patients and 37 environmental isolates collected in and around 7 deployed field hospitals in the Central Command area of responsibility. These isolates were referred to WRAIR for genetic analysis. The results of this EPICON were published in the journal *Clinical Infectious Diseases*.[21]

Genetic Analysis, Characterization, and Identification of the Source

Molecular epidemiology was performed on the clinical and environmental *Acinetobacter* isolates using PFGE and 16S rDNA sequencing in a single-blinded study.[21,45] A total of 201 of the 207 isolates were identified using 16S rDNA sequencing. The clinical isolates were almost evenly split between *A baumannii* and other ACB organisms. In contrast, only 19% of the environmental isolates were *A baumannii*, and 70% were ACB organisms other than *A baumannii*. PFGE was able to establish 66 clinical isolate clusters and 25 different environmental isolate clusters when clusters were defined as greater than 90% identical. Three different PFGE clusters contained isolates from clinical samples and the environmental isolates that were 100% identical. The matching environmental and clinical isolates were obtained from Camp Dogwood and WRAMC; LRMC, WRAMC, USNS *Comfort*, and Field Hospital Baghdad; and Mosul, Camp Dogwood, and LRMC. Additionally, two isolates from LRMC were 100% identical to an ACB isolate from WRAMC.[21] Taken together, these results strongly indicated that the outbreak of MDR *Acinetobacter* infections seen in the larger military medical centers began as nosocomial infection that originated in CSHs in Iraq. In essence, the MHS had become "infected" with *Acinetobacter* because patients who were not involved in Iraqi military operations became infected with the organisms.

Military and civilian casualties from OIF were evacuated to the United States and the United Kingdom. Hospitals in the United Kingdom reported an outbreak of *Acinetobacter* infections soon after OIF commenced. Initially, there was concern that the infections caused by *Acinetobacter* may have been due to an intentional release of the organism. To determine if the isolates obtained in the United Kingdom were similar to those found in US casualties; the two countries initiated a collaboration and analyzed representative strains.[46] The laboratory at WRAIR chose representative isolates from all of the major clusters that had been identified by PFGE and supplied them to the UK investigators. The UK Laboratory of HealthCare Associated Infections compared their *Acinetobacter* isolates to the ones supplied by the WRAIR laboratory. PFGE revealed that three of the US isolates were similar to the UK isolates with greater than 90% similarity. The antibiotic susceptibility profiles among these three isolates were

also similar. DNA sequence analysis of the integron region associated with antibiotic resistance in *Acinetobacter* revealed that two of these common isolates had identical sequences. The third common isolate revealed a duplication of one gene in the US isolate but was otherwise identical to the UK isolate. These results suggested that there was a common origin for the *Acinetobacter* isolates causing wound infections in both the United States and the United Kingdom.

Research, epidemiological investigation, and molecular typing indicated that the *Acinetobacter* infections were nosocomial such that casualties were becoming infected in theater, then the organism was becoming disseminated through the medical evacuation chain. A study utilizing multilocus PCR and mass spectroscopy (MS) was undertaken to analyze how the genotype of the organism might change over time in an MTF. A total of 267 *Acinetobacter* isolates were analyzed; 216 of the isolates were isolated from 2002 through 2004 and were part of the original EPICON previously analyzed by PFGE.[21,47] The additional isolates in this study were obtained from the American Type Culture Collection and from European hospitals. *A baumannii* accounted for 83% of the total isolates and could be divided into 46 unique sequence types (STs). This study also showed a strong correlation between isolates obtained from US OIF casualties and isolates obtained from European hospitals. Although the resolution of the PCR MS technique was lower than PFGE, there was good correlation between the two molecular methods. Additionally, this group analyzed the change in genotype of the *Acinetobacter* over time at WRAMC using PCR MS.[18] This study compared the ST of isolates obtained from 2002 to 2004 with the ST of strains isolated from 2006 to 2007 at WRAMC. The STs were relatively constant; a few minor STs either disappeared or increased with time, yet the major STs remained constant. Comparison of STs with nonmilitary hospital isolates revealed the distribution of STs was markedly different between the two groups. The antibiotic susceptibility profile generally correlated with ST as well. The study suggested that the *Acinetobacter* population in WRAMC had become less diverse and more stable with time. This was possibly due to effective countermeasures, such as sanitation and specific early therapy that resulted in reduction of less fit *Acinetobacter* strains both in patients and in the environment.

Antibiotic Resistance

MDR in *Acinetobacter* increased with time, making it more difficult for the MHS to respond to the threat. The genus is known as an opportunistic pathogen that resides in the environment and is naturally resistant to many antibiotics[35,48]; however, the organism also responds to antibiotic treatment by acquiring antibiotic-resistant genes. Interestingly, isolates obtained at BAMC from deployed service members were generally more resistant than those from nondeployed personnel.[49] Resistance to the drug of choice, imipenem, increased as casualties from OIF continued. Several studies have looked at which genes are involved in the MDR phenotype of *Acinetobacter* involved in military wound infections.

The study by Hujer et al used antibiotic-resistant, gene-specific PCR to analyze selected isolates from WRAMC to determine which MDR-associated genes the strains harbored.[50] Approximately 20% of the 75 isolates they analyzed were resistant to imipenem. Many isolates encoded multiple genes that were responsible for resistance to a class of antibiotic. Almost all of the isolates were resistant to ciprofloxacin through chromosomal housekeeping gene mutation. The strains were also highly resistant to cephalosporins by the production of beta-lactamase belonging to seven different classes of genes. Ninety percent of the imipenem-resistant isolates encoded the *bla*OXA-23 beta-lactamase allele. In another study, based on microarray analysis of antibiotic resistance genes found in 102 *Acinetobacter* isolates obtained from the National Naval Medical Center (NNMC) in 2006, 93% of the imipenem-resistant isolates were found to encode the *bla*OXA-23 beta-lactamase.[51] All of the imipenem-resistant isolates belonged to one of two PFGE clusters. Accordingly, the increase in imipenem resistance in *Acinetobacter*-colonizing OIF casualties was mostly due to acquisition of a single allele of beta-lactamase. The *bla*OXA-23 gene has been shown to be associated with bacterial mobile genetic elements allowing for rapid resistance acquisition and spread.

MILITARY HEALTH SYSTEM RESPONSE TO *ACINETOBACTER* AND OTHER "ESKAPE" PATHOGENS

Following the EPICON described above, many clinicians, scientists, and microbiologists recognized the use and value of establishing a centralized laboratory for receiving and archiving multidrug-resistant organisms (MDROs). Some of the major referral centers, such as NNMC, BAMC, and WRAMC, had already been preserving some MDR isolates, especially *Acinetobacter*, recognizing their inherent scientific and epidemiologic value. However, there was no central and standardized repository. Additionally, the institutions lacked the necessary human and financial resources to fully characterize these isolates. Centralized collection,

comprehensive characterization, and long-term storage of MDROs is essential to understanding the healthcare challenges and informing future approaches. The Agency for Healthcare Research and Quality estimates that 5% to 10% of all inpatients acquire one or more HAIs during their stay, at an annual cost of $28 to $33 billion. Twenty percent of HAIs are considered preventable through surveillance programs; however, as of late 2008, no agency in the Department of Defense was performing what would become the mission of the MDR Organism Repository and Surveillance Network (MRSN): to conduct enterprise-wide epidemiologic surveillance of MDROs to inform clinical practice and healthcare policy and enhance infection control.

The idea for a repository ("Joint Bacterial Repository") was presented to the leadership of the US Army Medical Research and Materiel Command in 2008; however, given the heightened sensitivity to biosurety amid the ongoing anthrax investigation involving scientists in the command, senior leaders were reluctant to authorize and establish what might be misperceived as another "freezer farm." A few months before, DoD-GEIS (now part of the Armed Forces Health Surveillance Center) had funded two small surveillance studies in Eastern Iraq during the troop surge of 2007.[52,53] These became the proof of concepts for a less static repository and more dynamic surveillance network. These studies demonstrated that regardless of location or environment, a distant facility could submit samples to a central laboratory and could receive useful and actionable information relating to infection control. The concept of a central repository laboratory at the nexus of a multifacility, bidirectional surveillance network (Figure 13-2) was successfully proffered to the US Army Medical Command (MEDCOM) in early 2009.

In June 2009, the bacterial diseases branch of WRAIR in Silver Spring, Maryland, launched the MRSN. Under a performance improvement mandate from MEDCOM, Army hospitals, including those in Iraq and Afghanistan, submit MDROs isolated from clinical infections and active surveillance efforts. Isolates undergo integrated phenotypic, clonal, and phylogenetic analyses, including high-resolution ordered whole genome restriction optical mapping, followed by archival cryopreservation. The MRSN works closely with the Centers for Disease Control and Prevention to ensure genotyping methods are the same. Repository personnel provide epidemiologic reports and infection control information to hospitals and policy makers, conduct site assistance visits, and post site-specific and global antibiograms on a secure website.[54,55] Other US military services are encouraged, but not required, to participate. Synergy is achieved through interagency collaboration and information sharing with the other military services, especially the Army's Pharmacovigilance Center and the Navy and Marine Corps Public Health EpiData center, and with international military and civilian colleagues.

Substantial evidence supports the MRSN's role in enhancing infection prevention and control efforts and reducing associated healthcare costs.[56–59] Authors of one outbreak investigation concluded that if an aggressive surveillance program had not been in place, the source of an outbreak of severe ventilator-associated pneumonia in Canadian soldiers caused by MDR *A baumannii* would have been missed.[60] The Association of Professionals in Infection Control and Epidemiology manual lists over 70 references demonstrating the usefulness of surveillance data in reducing infection occurrence and supporting the use of surveillance data to improve the quality of healthcare outcomes and processes.[61] McQuillen et al cited more than 20 studies quantifying the financial impact of HAIs and the cost savings associated with surveillance.[62] Infection control component monitoring and feedback, the approach taken by the MRSN, has been key in reducing the rate of HAIs (see Figure 13-2).[63,64] Choosing the correct therapy requires knowledge of the underlying disease, the previous patient colonization, and the microbial trends in the community, as well as in the specific healthcare facility. Submitting isolates to the MRSN makes the latter two possible by enabling the MRSN to produce facility-specific and regional antibiograms. Using that modus, the MRSN has achieved notable firsts in the MHS and, in 2010, won first place in the Army Surgeon General's Excalibur Award for health innovation practices. In 2012, the MRSN received accreditation by the College of American Pathologists (CAP), and in 2013 it won the Military Health System Award for Healthcare Innovation.

Currently more than 30 hospitals, including some in unstable areas and war zones, request molecular assistance with outbreak investigation or submitted isolates (an average of 375 per month). Isolates are identified and their susceptibility tested with the three most commonly used commercial automated susceptibility testing systems (VITEK 2 [bioMérieux, Inc, Durham, NC]; MicroScan [Siemens Medical Solutions, Malvern, PA]; and Phoenix [Becton, Dickinson, and Company, Franklin Lakes, NJ]). Discordant results of susceptibility testing are resolved by use of microbroth dilution panels. These data enable direct comparison of the three systems across multiple antibiotics and organisms, ranging from 8 drugs for *A baumannii* to 14 drugs for *Escherichia coli*, *Klebsiella pneumoniae*, and methicillin-resistant *Staphylococcus aureus*.

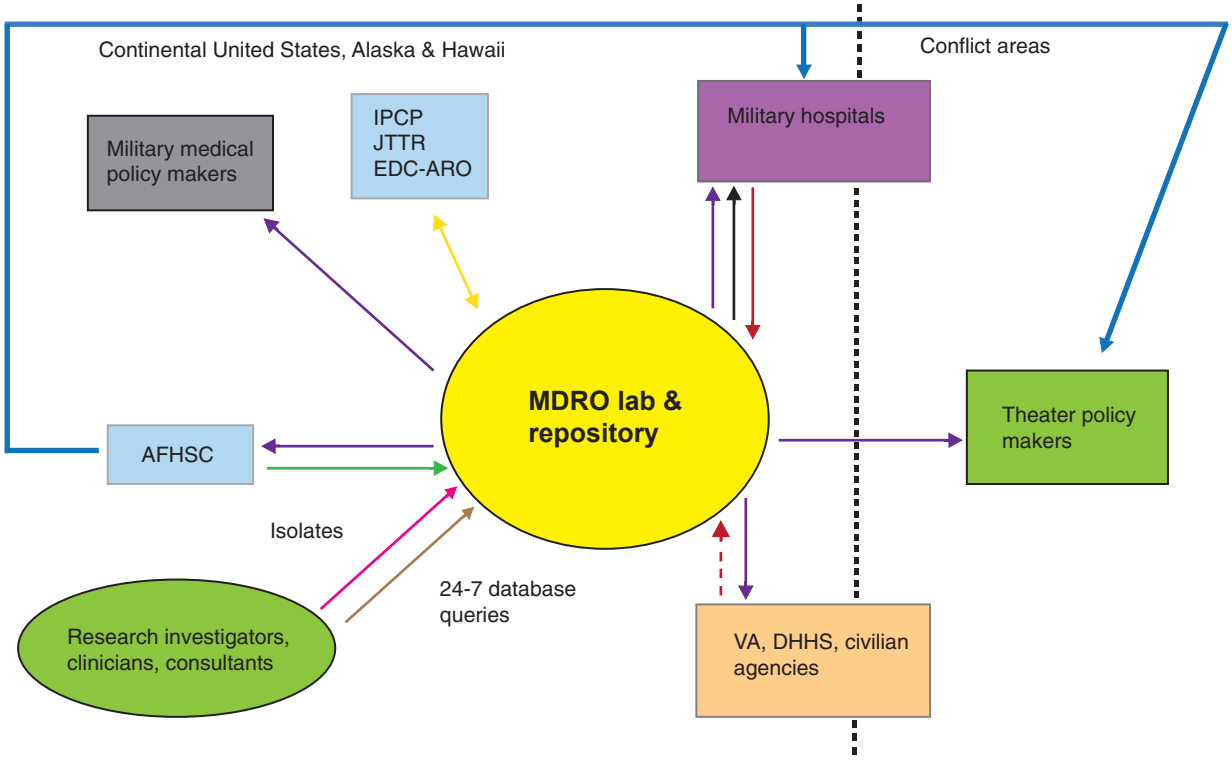


Figure 13-2. The *purple-shaded square* represents medical treatment facilities (Roles 3, 4, and above), which submit isolates and associated clinical data (*red arrow*) to the repository at Walter Reed Army Institute of Research (WRAIR; *yellow oval*) and in turn receive reports and assistance from WRAIR (*black arrow*). Reports and guidance will also be provided to theater medical leaders and combatant commanders (*green box via purple arrow*). Bidirectional information sharing and technical assistance can occur between the repository and other agencies, such as the Infection Prevention and Control Panel, the Joint Theater Trauma Registry, and the Navy's Public Health EpiData Center Antibiotic Resistance Organization (EDC-ARO; *blue box via yellow arrow*) to enhance surveillance and reports. Data and reports from the repository will also be forwarded to the Armed Forces Health Surveillance Center (AFHSC; *purple arrow*) for broad dissemination (*via blue arrows*); the AFHSC can also assist with epidemiologic analysis or generation of the final reports (*green arrow*). Reports and information can also be provided to consultants for microbiology, infectious diseases, surgery, trauma, the surgeons general and others (*gray box via purple and blue arrows*). Consultants, policy makers, clinicians, and research investigators will be able to query certain portions of the database through web-enabled architecture (*olive-tan arrow*). Investigators can request de-identified isolates and specimens for approved research protocols (*pink arrows*).
AFHSC: Armed Forces Health Surveillance Center
DHHS: US Department of Health and Human Services
EDC-ARO: EpiData Center Antibiotic Resistance Organization
IPCP: Infection Prevention and Control Panel
JTTR: Joint Theater Trauma Registry
VA: US Department of Veterans Affairs

Unique to MRSN, associated clinical and demographic information, including securely maintained personal identifiers, is also submitted, helping to avoid isolate duplication when producing antibiograms and enabling MDRO tracking across regions. Isolates without this information are collected, but their ability to inform infection control efforts is severely degraded.

The MRSN developed and validated multiplex real-time PCR platforms to test isolates for the presence of clinically important antimicrobial resistance genes, such as *mupA*, *qac*A/B, *PVL*, *cfr*, and *etA* in *Staphlococcus* species; all variants of *bla*NDM and *bla*KPC, and the most common variants of *bla*VIM and *bla*IMP, and the most relevant alleles of *bla*OXAs in carbapenem-resistant gram-negatives. These are usually plasmid-borne genes that encode for high-level mupirocin resistance (*mupA*), chlorhexidine tolerance or resistance (*qac*A/B), or carbapenemase production (*bla*KPC, *bla*NDM, *bla*VIM, *bla*IMP, and certain *bla*OXAs). They contribute to the dissemination

of biocide or antibiotic resistance. *Bla*NDM1 carried on a novel plasmid was detected in *Providencia stuartii* from Afghanistan.[65] The MRSN was first to report *qac*A/B in methicillin-resistant *Staphylococcus aureus* in the United States, which had higher tolerance of chlorhexidine gluconate than those that did not have the gene.[66] A cluster of colistin-resistant *Acinetobacter* emerged during therapy with colistin. This clone or strain, MLST 94, appeared to have a tendency to develop colistin resistance rapidly upon exposure to colistin, so the MRSN developed an assay capable of detecting the genetic element associated with that strain. It combined optical mapping and sequencing to identify gene copy number changes in sequential *Acinetobacter* isolates from the same patient.[67] The MRSN also improved the speed and reduced the cost of optical genome mapping by successfully mapping multiple genomes on the standard map card.[68]

The MRSN provided the foundation for enhanced *de novo* assembly of high-throughput pyrosequencing data using optical genome mapping, and demonstrated the first clinically relevant application of next-generation sequencing in the MHS.[69] It also allowed identification of a heretofore notoriously difficult-to-characterize species of *Acinetobacter* that is typically misidentified by commercially available identification systems.[70] The intent is that the MRSN become a reference laboratory for DoD-GEIS for high-resolution characterization of the MDR ESKAPE pathogens, with the ultimate goal of becoming the first CAP-accredited laboratory for optical mapping and sequencing in the DoD. Aligned with the One Health philosophy, the MRSN also supports or performs canine and environmental surveillance. Currently the MRSN has collaborators in 32 hospitals from 12 countries in Central and South America, Europe, Asia (including Iraq and Afghanistan), and North America. The demonstrable power of surveillance increases in proportion to the geographic area surveyed and the degree of sharing between those who need to know and those who can act on findings to improve patient safety.[61] To that end, the MRSN continually invites more international civilian and military colleagues to collaborate and submit isolates.

The Way Forward

Current typing technologies have been useful in revealing relationships between isolates of ACB, but they are unable to resolve differences between closely related isolates from small-scale outbreaks, where chains of transmission are often unclear. Increasingly, genome scale epidemiology is required to detect and respond to outbreaks of highly resistant and virulent bacterial "superbugs."[71–73] Recall the recent fatal outbreak at the National Institutes of Health and the amount of sequencing and computing power that was needed to determine the origination and pattern of spread.[72] Another study investigated a polyclonal outbreak of MDR *A baumannii* using whole genome sequencing. Comparison of the complete genome sequences of three dominant outbreak strain types showed that these strains diverged before their arrival at the authors' institution despite all belonging to the same epidemic lineage (International Clonal Complex II).[72] The simultaneous presence of three divergent strains of the same lineage is in accordance with its increasing prevalence in international hospitals, further supporting the ongoing adaption of clonal complex II to the hospital environment.[72,74] Finally, a recent study found that nearly every strain of ACB from one integrated hospital system in the United States was unique despite being indistinguishable by conventional sequence typing methods, and in some cases by core single nucleotide variation typing.[73]

The recent availability of rapid and inexpensive whole genome sequencing permits detailed investigation of genetic differences between bacterial isolates belonging to a single species and gives insight into the nature of genetic changes between isolates under antibiotic selection pressure. This is the approach now taken by the MRSN, and has been used to elucidate the evolutionary origin of an outbreak of colistin-resistant *A baumannii* containing a novel operon, the genomic characterization of a separate clone of *Acinetobacter* responsible for a group of fatal infections, and a new strain type in Honduras.[2,75] Overall, as whole genome sequencing technologies continue to improve with respect to price and speed, these approaches should become the gold standard for rigorous epidemiological analysis in variable circumstances ranging from single-hospital outbreaks to worldwide epidemics, and from retrospective analysis to real-time monitoring.

Applying basic and translational research methods to improve surveillance conducted for quality improvement and infection control resulted in the evolution of the MRSN into the Antimicrobial Resistance Monitoring and Research (ARMoR) Program. It is an enterprise-wide collaboration to aid in infection prevention and control. This approach consists of a network of epidemiologists, bioinformaticists, researchers, policy makers, and hospital-based infection preventionists who collaborate to collect relevant antimicrobial resistance monitoring (ARM) data, conduct centralized molecular characterization, and use ARM characterization feedback to implement ap-

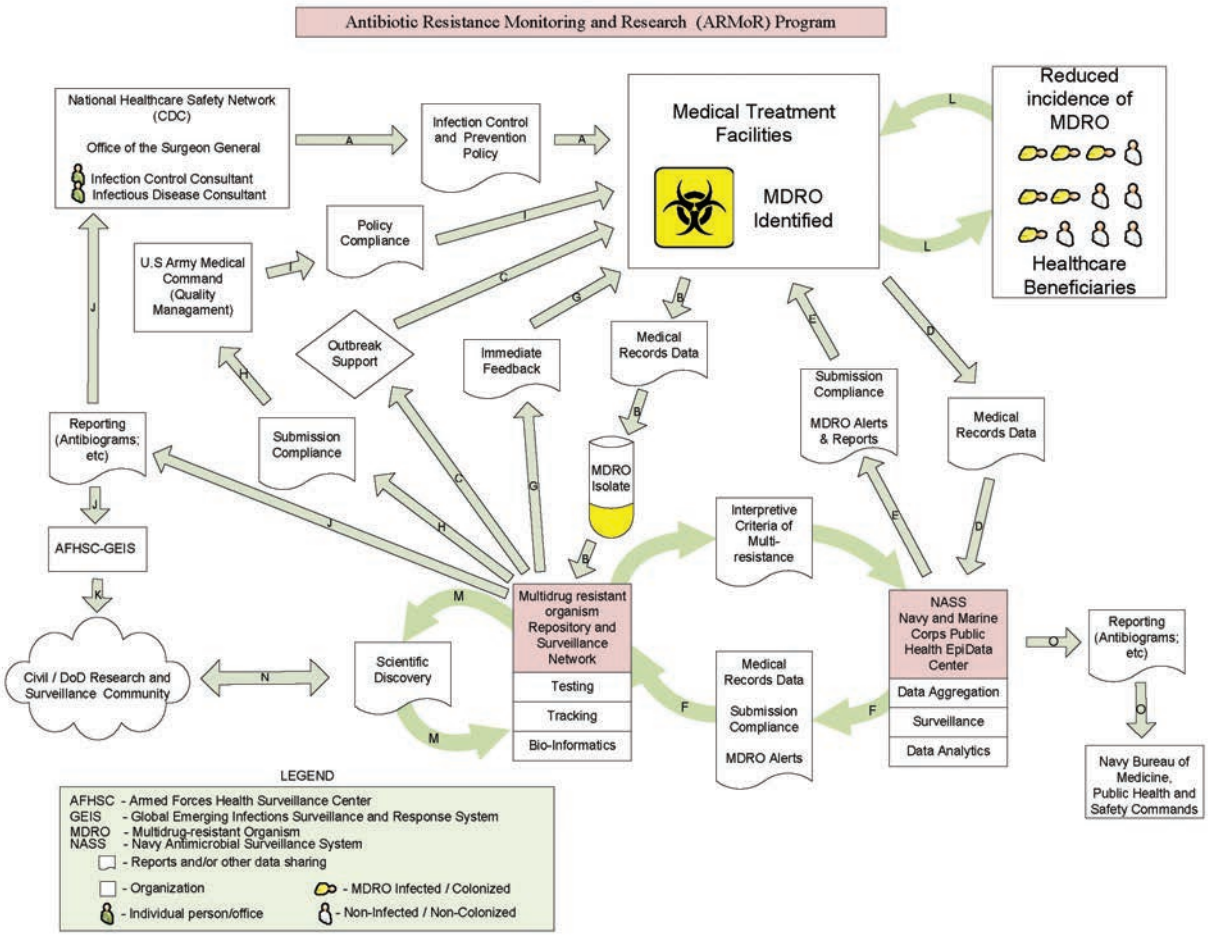


Figure 13-3. The Department of Defense Antibiotic Resistance Monitoring and Research Program (ARMoR) isolate and data flow.

propriate infection prevention and control measures. Policy compliance and enforcement further distinguish the ARMoR Program from the MRSN (Figure 13-3). A particularly concerning type of ARM, carbapenem-resistant *Enterobacteriaceae*, peaked immediately after the program was launched, then significantly declined. Similarly, there have been no further reports or outbreaks of another concerning type of ARM, colistin resistance in *Acinetobacter*, in the DoD since the program was initiated.

SUMMARY

The MHS response to *Acinetobacter* exemplifies how different parts of the MHS coming together to investigate and respond to a major threat to wounded warriors and all MHS beneficiaries. Infectious disease physicians recognized the problem early and engaged DoD medical leadership to task public health professionals with identifying the problem so actions could be taken to stop these costly infections. Public health professionals took advantage of laboratory expertise that had been initially developed for the DoD chemical and biological defense program to perform molecular analysis to determine genetic relationships among the *Acinetobacter* isolates. This fact underscores the need for collaboration between the Chemical Biological Defense Program and that part of the MHS that conducts "routine" infectious disease and public health investigations. Such collaboration supports service members and enhances the MHS's ability to respond during crises.

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Chapter 14

BOTULINUM TOXIN

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INTRODUCTION

HISTORY

DESCRIPTION OF THE AGENT
- **Botulinum Neurotoxin Production**

PATHOGENESIS

CLINICAL DISEASE

DIAGNOSIS
- **Diagnostic Assays in Botulism**
- **Foodborne Botulism**
- **Inhalation-acquired Botulism**
- **Other Forms of Botulism**

TREATMENT
- **Antitoxin**
- **Clinically Relevant Signs of Bioterrorist Attack**
- **Preexposure and Postexposure Prophylaxis**
- **New Vaccine Research**
- **New Therapeutic Drug Research**

SUMMARY

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A portion of this chapter has previously been published as: Dembek ZF, Smith LA, Rusnak JM. Botulism: cause, effects, diagnosis, clinical and laboratory identification, and treatment modalities. *Disaster Med Public Health Prep*. 2007;1(2):122–134.

INTRODUCTION

The seven neurotoxin serotypes (A-G) produced by *Clostridia* species are among the most potent toxins known. These structurally similar neurotoxins are immunologically distinct because neutralizing antibodies for one serotype does not protect against the other six serotypes.[1] Because of their extreme toxicity, neurotoxin from *Clostridia botulinum* was one of the first agents to be considered as a biological weapons agent. Botulinum neurotoxin has been developed as a biological weapon by many countries, including Japan, Germany, the United States, Russia, and Iraq (Figure 14-1).

Botulism in its various forms (foodborne, wound, infant and adult intestinal, and iatrogenic) is a potentially fatal neuroparalytic disease that most often presents as a descending, symmetric flaccid paralysis,[2] which is typically associated with neurotoxin types A, B, and E. Foodborne outbreaks receive considerable public health attention, as the risk of widespread food dissemination of botulinum toxin constitutes a public health emergency and is often fatal if untreated. However, the most common form of botulinum intoxication in the United States is infant botulism from the intestinal colonization with toxin-producing *Clostridium* in infants younger than 1 year of age.[3] For a recent 10-year period (2002–2011), of 1,379 reported cases of botulism in the United States, the greatest number was infant botulism (68%), followed by wound botulism (18%), and foodborne botulism (12%).[4]

HISTORY

In the early 1930s during its occupation of Manchuria, Japan formed a biological warfare research program. The largest facility in this program, which was located in Pingfang, was known as Unit 731.[5] General Shiro Ishii, the Japanese military medical commander of Unit 731, admitted to feeding lethal cultures of *C botulinum* to prisoners.[6] US researchers began working on weaponization of botulinum toxin in the 1940s, and Allied intelligence indicated that Germany attempted to develop botulinum toxin as a weapon to be used against invasion forces.[7] At the time, neither the composition of the toxic agent produced by *C botulinum* nor its mechanism of injury were fully known. Therefore, the earliest research goals were to isolate and purify the toxin and determine its pathogenesis,[8,9] with the latter work conducted at Camp Detrick. The potential of botulinum neurotoxin as an offensive biological weapon was also investigated.[10–12] The US code name given to botulinum neurotoxin at that time was "agent X."

Following President Richard M Nixon's executive orders in 1969–1970, as explicitly stated in National Security Decision Memoranda 35 and 44,[13] all biological agent stockpiles in the US offensive biological program, including botulinum neurotoxin, were destroyed.[14] The 1975 Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction prohibited the production of offensive toxins.[15] Although the Soviet Union signed and ratified this convention, its biological warfare program, which included botulinum neurotoxin research, biological weapons development, and production, continued and was expanded in the post-Soviet era.[16,17] The Soviet Union reportedly tested botulinum-filled weapons at the Soviet site Aralsk-7[16] on Vozrozhdeniye (Renaissance) Island in the Aral Sea[17,18] and attempted to use genetic engineering technology to transfer complete toxin genes into other bacteria.[19] In April 1992, President Boris Yeltsin publicly declared that his country had covertly continued a massive offensive

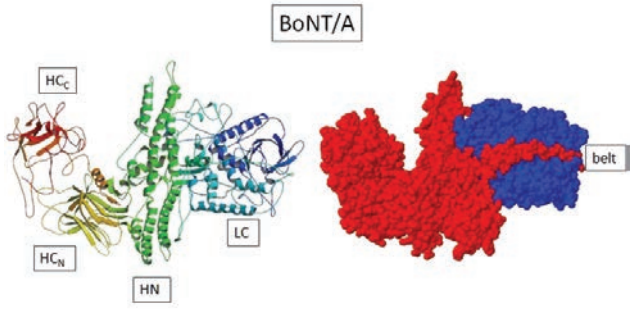


Figure 14-1. Representations of the structure of BoNT/A. *Left panel*: the protein is composed of a ~50 kDa light chain (LC, blue-green) and a ~100 kDa heavy chain. The heavy chain is composed of three distinct structural domains: two C-terminal ganglioside binding domains are used in recombinant vaccines, HC_c (red) and HC_n (yellow), and an N-terminal translocation domain (HN, green). The LC functions as a zinc-dependent endopeptidase. *Right panel*: in this rendering of the molecule, the belt-like portion of the heavy chain (red) is more clearly seen to wrap around the LC (blue). Data sources: (1) Lacy DB, Tepp W, Cohen AC, et al. Crystal structure of botulinum neurotoxin type A and implications for toxicity. *Nat Struct Biol*. 1998;5:898–902. (2) Swaminathan S, Eswaramoorthy S. Structural analysis of the catalytic and binding sites of *Clostridium botulinum* neurotoxin B. *Nat Struct Biol*. 2000;7:693–699.

biological warfare buildup, which included developing botulinum toxin as a weapon. Yeltsin's assertions gave credence to the claims of Biopreparat laboratory director Vladimir Pasechnik, who defected to the United Kingdom in 1989.[20] Also in 1992, Colonel Kanatjan Alibekov (Kenneth Alibek),[19] the former deputy director of Biopreparat (a Soviet agency whose primary function was to develop and produce biological weapons of mass casualties), defected to the United States and eventually described in considerable detail the Soviet biological weapons program in his book *Biohazard*.[19]

Iraq, which also signed the 1975 convention, expanded its biowarfare program in 1985. Ten years later, it admitted to the United Nations Special Commission inspection team to having produced 19,000 liters of concentrated botulinum neurotoxin for use in specially designed missiles, bombs, and tank sprayers in 1989 and 1990.[16,21] Of this preparation, 10,000 liters were used to fill 13 SCUD missiles with a 600-km range and 100 181-kg (R-400) bombs (each bomb could hold 83 liters of toxin solution). However, Iraq did not use biological agents during the Persian Gulf War or Operation Iraqi Freedom, and it has maintained that its biological weapon stockpiles were destroyed.[22] No biological weapons were discovered in Iraq in wartime from 2003 through 2011.[23]

The Aum Shinrikyo, a Japanese cult formed in 1987 by Shoko Asahara, attempted to develop biological weapons after its political party was defeated in the 1990 election campaign. Known for its deadly 1995 sarin attack in the Tokyo subway, Aum Shinrikyo also attempted to produce botulinum neurotoxin. Five days before the sarin attack, three briefcases containing portable disseminating devices generating water vapor were found in the Kasumigaseki subway station.[24] At his 1996 trial, Asahara said he believed the cases contained botulinum neurotoxin, although the toxin was not detected in the devices. With 50,000 followers worldwide and an estimated $1 billion in financial resources, the cult had the capability to develop biological toxins for use as weapons, and the intent to do so.[25] Although no cult members were specialists in biological weapons development, microbiologists, medical doctors, and other scientists were among the followers. In their thorough analysis of the Aum Shinrikyo's efforts to develop biological and chemical weapons, Richard Danzig and his colleagues postulate that the group specifically failed to develop a viable botulinum toxin because of the following reasons[24]:

- inability to acquire an appropriate strain of *C botulinum* capable of producing sufficient concentrations of active botulinum toxin (five different strains were isolated by the cult);
- inability to achieve specific culture conditions (eg, appropriate fermentation broth, appropriate anaerobic environment) required for toxin production;
- presence of bacterial contamination, as *Bacillus subtilis natto* was identified in the cult's *C botulinum* product (as an aerobic bacteria, also indicates inappropriate anaerobic cultural conditions);
- toxin degradation that may have occurred during postfermentation recovery or storage; and
- suboptimal concentrations of toxin (if present in any amount) that were disseminated that did not cause harm.

A successful bioterrorist attack on large numbers of people with botulinum neurotoxin would overwhelm the public health system. The medical intervention required to assist patients with botulism includes mechanical ventilation and urgent attendant healthcare. If the Rajneeshee cult (the followers of the Bhagwan Shree Rajneesh who had carried out a biological attack to influence a local election) had used botulinum toxin instead of *Salmonella typhimurium* on salad bars in its 1984 attack in The Dalles, Oregon,[26–28] many of the 751 persons who contracted Salmonella gastroenteritis would likely have died; the neurological sequelae of hundreds of patients with botulinum toxin poisoning would have quickly overwhelmed community medical resources.[29]

The potential consequences of a botulinum toxin-induced mass casualty disaster can be estimated by reviewing the March 2006 foodborne botulism outbreak in Thailand.[30] This event signifies a profound national public health mass casualty event, and successful patient recovery is not trivial. In this outbreak, home-canned bamboo shoots were consumed in a village, where 209 individuals consumed a common meal. Of 163 individuals examined in a hospital, 140 were hospitalized and 42 developed respiratory failure and required mechanical ventilation.[30] Sufficient antitoxin was donated to Thailand by various international health organizations to treat 90 patients. This antitoxin was administered to patients with the most severe symptoms, but treatment was delayed 5 to 9 days from exposure. The median duration of hospital admission was 6 days for patients without mechanical ventilation and 25 days for patients with mechanical ventilation.[31] A long incubation time was associated with a better prognosis.[32] The massive public health response undertaken by the Thai healthcare system, the global health community, and others outside of healthcare, including many embassies, airlines, and commercial

partners, is credited for no mortalities.[30] Besides the need for adequate ventilators and healthcare support, successful treatment of such large numbers of severely affected patients required intervention from neurologists, pulmonologists, intensivists, cardiologists, infectious disease specialists, and rehabilitation and referral services.[31] Other supportive personnel required for a mass casualty botulism event included pharmacists, respiratory care officials, and psychological services personnel.[33,34]

In 2005, Wein and Liu[35] described in detail how a bioterrorism attack using botulinum neurotoxin could be perpetrated on the nations' milk supply. They describe a mathematical model representative of California's dairy industry with milk traveling from cows to consumer in a supply chain: milk is processed from cows; picked up by tanker truck; piped through milk silos; processed via separation, pasteurization, homogenization, and vitamin fortification; and eventually distributed to the public.[35] Naturally occurring salmonellosis outbreaks from milk and milk products affecting more than 200,000 persons have occurred, leading to a realistic assessment of such vulnerability in the national milk distribution system.[36,37] The ability to spread botulinum neurotoxin via a liquid media, if present in sufficient concentration, makes this agent a logical choice for such a scenario. Modeling of botulinum in a liquid dispersal medium is not new and has been posited for terrorist use in a water fountain,[38] based on microbiological contamination at a recreational facility.[39] However, Wein and Liu's modeling goes further than tocsin generation, pinpointing critical entry points of neurotoxin into the milk supply, estimating the amount of toxin required, and identifying weaknesses in current detection technology.[35,39] The Wein and Liu paper generated considerable debate[40] by the possible security risk it exposed.[41] Stewart Simonson, former assistant secretary for public health emergency preparedness at the US Department of Health and Human Services, regrets the publication decision.[42] It has been demonstrated that the milk supply is likely not at a risk for botulism contamination during the presterilization process because standard pasteurization at 72°C for 15 seconds inactivates at least 99.99% of BoNT/A and BoNT/B and at least 99.5% of their respective complexes.[43]

DESCRIPTION OF THE AGENT

Clostridium species bacteria are sporulating, obligate anaerobic, gram-positive bacilli. The spores of *C botulinum* are ubiquitous, distributed widely in soil and marine sediments worldwide, and often found in domestic grazing animals' intestinal tracts.[44–48] Under appropriate environmental or laboratory conditions, spores can germinate into vegetative cells that will produce toxin. *C botulinum* grows and produces neurotoxin in the anaerobic conditions frequently encountered in food canning or preservation. The spores are hardy, and special efforts in sterilization are required to ensure that the spores are inactivated.[49] Modern commercial procedures have virtually eliminated food poisoning by botulinum toxin. However, the leading cause of foodborne botulism is attributed to home-canned foods (particularly vegetables such as beans, garlic, peppers, carrots, and corn that are pH >4.6) or food items improperly prepared by restaurants.[50–52]

C botulinum produces eight antigenic types of neurotoxins denoted by the letters A through H. Seven neurotoxins (A-G) are structurally similar (approximately 150 kD in mass) but immunologically distinct.[53,54] However, there is some serum cross-reactivity among the serotypes because they share some sequence homology with one another as well as with tetanus toxin.[55,56] The unique strain *Clostridium baratii* produces only serotype F,[57] and the *Clostridium butyricum* strain, serotype E.[58]

Botulism is a neuroparalytic disease. Human botulism cases are caused primarily by neurotoxin types A, B, and E,[50] and rarely by type F.[59] *Clostridium argentinense* produces type G, which has been associated with sudden death, but not neuroparalytic illness, in a few patients in Switzerland.[60] Neurotoxin types C and D cause disease in animals. All seven toxins are known to cause inhalational botulism in primates,[61] and therefore could potentially cause disease in humans. Clostridial C2 cytotoxin is a nonhomologous enterotoxin, and not a selective neurotoxin.[62] It affects multiorgan vascular permeability via cellular damage from its action on actin polymerization in the cellular cytoskeleton, and has been implicated in a fatal enteric disease in waterfowl.[63,64] Although the newly described botulinum neurotoxin (H) is structurally related to serotypes A and F, it is not a distinct serotype because it has been demonstrated to be neutralized with serotype A antitoxin.[65] A gene sequence of two botulinum toxin gene clusters differed substantially from the sequences of BoNT genes for toxins A-G.[55]

Botulinum Neurotoxin Production

Spore germination and subsequent growth of toxin-producing bacteria occur in improperly preserved foods,[66–72] decaying animal carcasses and vegetable

matter,[73–77] and microbiology laboratories.[78–82] A terrorist with the proper expertise and resources could obtain a toxin-producing strain of *C botulinum*. Various scientific journals, textbooks, and Internet sites provide information on how to isolate and culture anaerobic bacteria and, specifically, how to produce botulinum toxin. A major cause of botulism is the ingestion of foods contaminated with *C botulinum* and preformed toxin. The food supply remains vulnerable to a botulinum toxin attack.

PATHOGENESIS

The seven neurotoxins have different specific toxicities[83–85] and durations of persistence in nerve cells.[86,87] All botulinum toxin serotypes inhibit acetylcholine release, but they act through different intracellular protein targets, exhibit different durations of effect, and have different potencies.[88] All seven toxins may potentially cause botulism in humans given a large enough exposure. Botulinum neurotoxin can enter the body via the pulmonary tract (inhalational botulism), the gastrointestinal tract (foodborne and infant botulism, adult intestinal [adult infectious] botulism), direct injection (iatrogenic botulism), and from infected wounds (wound botulism). Infant, adult intestinal, and wound botulism are also referred to as infective botulism, as toxin produced by *C botulinum* either colonizes the lumen of the intestinal tract or is produced in a wound.[89] Upon absorption, the circulatory system transports the toxin to peripheral cholinergic synapses, primarily targeting neuromuscular junctions.[90] The toxin binds to high-affinity presynaptic receptors and is transported into the nerve cell through receptor-mediated endocytosis. In the nerve cell, it functionally blocks neurotransmitter (acetylcholine) release, thereby causing neuromuscular paralysis. Other neurotransmitters co-located with acetylcholine may also be inhibited,[91,92] and noncholinergic cells may also be affected.[93] The estimated human dose (assuming a weight of 70 kg) of type A toxin lethal to 50% of an exposed population (the LD_{50}) is estimated, based on animal studies, to be approximately 0.09 to 0.15 μg by intravenous administration, 0.7 to 0.9 μg by inhalation, and 70 μg by oral administration.[94–97]

CLINICAL DISEASE

Untreated botulism is frequently fatal. The rapidity of the onset of symptoms, as well as the severity and duration of the illness, is dependent on the amount and serotype of toxin.[50,98] In foodborne botulism, symptoms appear several hours to within a few days (range 2 hours to 10 days) after contaminated food is consumed.[50,99] In most cases the onset of symptoms occurs within 12 to 72 hours postexposure. In one study, the median incubation period for the onset of symptoms from all toxin serotypes was 1 day.[98] However, the median time to onset of symptoms for serotype E was shorter (range 0–2 days) compared to toxin serotypes A (range 0–7 days) and B (range 0–5 days); most individuals with toxin serotype E had symptoms within 24 hours of ingestion. Symptoms from foodborne botulism from toxin serotype A generally are more severe than from toxin serotypes B and E.[98]

As a neuroparalytic illness, botulism presents as an acute, symmetrical, descending, and flaccid paralysis. However, early symptoms may be nonspecific and difficult to associate with botulinum intoxication. Individuals with foodborne botulism often present initially with gastrointestinal symptoms such as nausea, vomiting, abdominal cramps, and diarrhea. Initial neurologic symptoms usually involve the cranial nerves, with symptoms of blurred vision, diplopia, ptosis, and photophobia, followed by signs of bulbar nerve dysfunction such as dysarthria, dysphonia, and dysphagia. Onset of muscle weakness ensues in the following order: muscles involving head control, muscles of the upper extremities, respiratory muscles, and muscles of the lower extremities. Weakness of the extremities generally occurs in a proximal-to-distal pattern, and is generally symmetric.[51] However, asymmetric extremity weakness may occasionally be observed, occurring in 9 of 55 botulism cases in one review.[100]

Respiratory muscle weakness can result in respiratory failure, which may be abrupt in onset. In one study, the median time between the onset of intoxication symptoms and intubation was 1 day.[98] Other commonly reported symptoms included fatigue, sore throat, dry mouth, constipation, and dizziness.[100] Botulism is not associated with sensory nerve deficits. However, one review of botulism from toxin serotype A or B showed that 8 of 55 cases reported symptoms of paresthesias.[100] Death is usually the result of respiratory failure or secondary infection associated with prolonged mechanical ventilation. In general, intoxication with toxin serotype A results in a more severe disease, often with bulbar and skeletal muscle impairment, and thus the need for mechanical ventilation.[98,100,101] Intoxication with toxin serotype B or E is more often associated with symptoms of autonomic dysfunction, such as internal ophthalmoplegia, nonreactive dilated pupils, and dry mouth.

Paralysis from botulism can be long lasting. Mechanical ventilation may be required for 2 to 8 weeks with foodborne botulism, with paralysis lasting as long as 7 months.[100] Symptoms of cranial nerve dysfunction and mild autonomic dysfunction may persist for more than a year.[102–104]

The following symptom triad should suggest a botulism diagnosis: (1) an acute, symmetric, descending, flaccid paralysis with prominent bulbar palsies in (2) an afebrile patient with (3) a normal sensorium. The bulbar palsies of botulism consist of the "four Ds": diplopia, dysarthria, dysphonia, and dysphagia. Five classic symptoms have also been used to diagnose botulism: (1) nausea and vomiting, (2) dysphagia, (3) diplopia, (4) dry mouth, and (5) fixed dilated pupils.[100] However, individuals may not exhibit all five symptoms; a review from the country of Georgia reported that only 2% of patients (13/481) presented with all five criteria.[72]

Epidemiological history of injection of black tar heroin (wound botulism), laboratory work with botulinum toxins, or therapeutic use (eg, for cervical dystonia or cosmetic purposes) of botulinum neurotoxin preparations not approved by the Food and Drug Association (FDA) may also support the diagnosis of botulism.[105–107] Patients with botulism may have a delay in diagnosis because of vague complaints or symptoms only present early in the illness or from misdiagnosis of paralytic symptoms.[108] In one study, less than half of the patients with botulism requiring hospitalization were diagnosed and admitted during the initial physician contact.[100] Alternate diagnoses may include drug overdose or intoxication (particularly with injection drug users having wound botulism) malingering, conversion disorder, stroke, myasthenia gravis, viral syndromes, and others.[108,109] Clinicians should be aware of early symptoms of botulism in adults (ie, blurred vision, diploplia, and/or ptosis) and infants. In addition, delays to diagnosis are associated with more prolonged hospital course and an increased need for ventilatory support.[108,110]

Although foodborne botulism is the most likely route of exposure for botulism from either natural causes or a bioterrorist event, botulism acquired on the battlefield is most likely to occur from botulinum toxin inhalation, a route of exposure that does not naturally occur. The duration from exposure to the onset of symptoms for inhalational botulism is similar to that observed with ingestion of botulinum toxin, generally ranging from 24 to 36 hours to several days postexposure.[98,111] Clinical symptoms resulting from inhalational intoxication are similar to botulism acquired from toxin ingestion.

The first reported inhalation-acquired botulism in humans occurred in 1962 in a German research laboratory.[112] Three laboratory workers experienced symptoms of botulinum intoxication after conducting a postmortem examination of laboratory animals that had been exposed to botulinum toxin type A. Hospitalized 3 days after their exposure, the workers were described as having (*a*) a "mucous plug in the throat," (*b*) difficulty in swallowing solid food, and (*c*) "the beginning of a cold without fever." The symptoms had progressed on the 4th day, and the patients complained of "mental numbness," extreme weakness, and retarded ocular motions. Their pupils were moderately dilated with slight rotary nystagmus, and their speech became indistinct and their gait uncertain. The patients were given antibotulinum serum on the 4th and 5th days. Between the 6th and 10th days after exposure, the patients experienced steady reductions in their visual disturbances, numbness, and difficulties in swallowing. They were discharged from the hospital less than 2 weeks after the exposure, with a mild general weakness as their only remaining symptom.[112] Botulism acquired through illicit drug (cocaine) inhalation has also been reported, and the incubation period was difficult to calculate because of frequent drug use.[113]

Other forms of botulism (wound, infant, and iatrogenic botulism) have similar symptoms as foodborne botulism, but they may have some variation in clinical presentation or clinical findings. Wound botulism is associated with an infected wound. However, the wound botulism associated with injecting drug use (usually an abscess) may not be grossly infected (15% to 50% cases), the wound site (usually at injecting sites) may be at an unusual location (ie, base of the tongue), and fever is generally not present.[108] The onset of symptoms in wound botulism associated with injecting drug use (mainly black tar heroin) has ranged from 2 to 14 days, and in wound botulism not associated with injecting drug use (ie, traumatic wounds) from 2 to 18 days (mean of 5 to 7 days).[105,108] Early symptoms in infant botulism are constipation or change in stool pattern, irritability, poor feeding, lethargy, and cranial nerve findings.[110] Cranial findings in infant botulism may initially be mild and overlooked, and generally manifest as a weak cry, poor suck or gag reflex, sluggish and incomplete pupillary response, ophthalmoplegia, and ptosis. Mortality from infant botulism with supportive intensive care (even before availability of antitoxin for infants) is generally less than 1%.[114] In iatrogenic botulism associated with therapeutic BoNT products (but not reported with lower doses given for cosmetic purposes), onset of symptoms generally occurred within hours to 3 weeks after the last injection.[108]

DIAGNOSIS

The differential diagnosis of botulism includes other diseases with paralysis symptoms[100]:

- Diphtheria (may cause paralysis but also exhibits fever, typical pharyngeal or nasal mucosal lesions, cervical adenopathy, cardiac conduction abnormalities).
- Guillain-Barré syndrome (usually ascending paralysis, paresthesias common, elevated cerebrospinal fluid [CSF] protein [may be normal early in illness], electromyogram findings). The CSF findings are usually normal in botulism, but mild elevation of CSF protein between 50 and 60 mg/dL has been noted in a minority of botulism patients. Most patients (90%) with the Miller Fisher syndrome variant of Guillain-Barré syndrome also have serum antibodies to the gangliosides GQ1b and GT1a.[115]
- Myasthenia gravis (dramatic improvement with edrophonium chloride, autoantibodies present, electromyogram findings). Botulism cases may have a positive response to edrophonium chloride (26%), but the response is generally not dramatic.
- Tick paralysis (ascending paralysis, paresthesias common, usually does not involve cranial nerves; detailed exam often shows presence of tick).
- Lambert-Eaton syndrome (commonly associated with carcinoma, particularly lung carcinomas; deep tendon reflexes absent; usually does not involve cranial nerves; electromyogram findings similar to botulism).
- Stroke or central nervous system mass lesion (paralysis usually asymmetric, brain imaging abnormal).
- Paralytic shellfish poisoning (history of shellfish ingestion; paresthesias of mouth, face, lips, and extremities common).
- Belladonna toxicity, such as atropine (history of exposure, tachycardia, and fever).
- Aminoglycoside toxicity (drug history of aminoglycoside therapy).
- Other neurotoxins, such as snake toxin (history of snake bite, presence of fang punctures).
- Chemical nerve agent poisoning (often associated with ataxia, slurred speech, areflexia, Cheyne-Stokes respiration, and convulsions).

A botulism diagnosis may be suggested by the clinical presentation of an afebrile patient with an acute, symmetric, descending, flaccid paralysis (without sensory deficits) with a normal sensorium. Any occurrence of botulism requires notification of public health officials and an epidemiological evaluation. Electrophysiological studies are helpful in distinguishing botulism from other causes of acute flaccid paralysis and support a presumptive diagnosis of botulism.[116–118] An electromyogram with repetitive nerve stimulation at 20 Hz to 50 Hz showing facilitation (an incremental response to repetitive stimulation), usually occurring only at 50 Hz, may be helpful in distinguishing botulism from Guillain-Barré syndrome or myasthenia gravis, but not from Lambert-Eaton syndrome.[51] Botulism patients with neuromuscular respiratory failure showed mostly incremental responses to high-rate repetitive nerve stimulation testing of the extensor muscles of the fifth digit in 17 patients, especially in response to >20 Hz stimulation.[119] Electrophysiological testing in botulism may also demonstrate a small evoked muscle action potential response to a single supramaximal nerve stimulus, with normal sensory nerve function and nerve conduction velocity test results. However, electrophysiological tests may be normal in botulism. Approximately 15% of patients with botulism may have normal muscle action potential amplitudes, and as many as 38% of patients may not exhibit facilitation.[100] CSF findings are usually normal in botulism, and abnormal findings should suggest another diagnosis. However, mild elevation of CSF protein (between 50 and 60 mg/dL) has been reported in 3 of 14 patients (21%) who had spinal fluid analysis performed.[98] Laboratory findings, such as complete blood count, chemistries, liver and renal function tests, and electrocardiogram, are normal in botulism, unless a complication (eg, secondary infection, respiratory failure) has occurred.

Diagnostic Assays in Botulism

A confirmatory diagnosis of botulism can often be made by demonstrating the presence of toxin in patient specimens, such as the serum, stool, gastric aspirate, vomitus, or wound, using mouse bioassays. Mouse bioassays, which are highly sensitive to botulinum toxin (0.01 ng/mL detection limit), are performed by injecting mice intraperitoneally with the specimen sample suspected to contain toxin (with and without various antitoxins). If toxin is present in the specimen, mice injected with the clinical specimen alone (without the specific antitoxin) will usually die from botulism within 6 to 96 hours, but mice injected with the specimen treated with the specific antitoxin will survive. Specimens for mouse bioassays may be sent

to the Centers for Disease Control and Prevention (CDC) or other designated state or municipal public health laboratories.[120]

Diagnosis can also be achieved by anaerobic culture and isolation of *Clostridium* species toxigenic strains from clinical specimens, including fecal specimens, gastric aspirates, vomitus, or infected wounds. The organism or toxin can also be isolated from the suspect food to help support the diagnosis.

In recent years the CDC has also used real-time polymerase chain reaction (PCR), Endopep-MS (mass spectrometry), and/or an enzyme-linked immunosorbent assay (A, B, E, F) to optimize its evaluation of botulism cases and outbreaks. However, real-time PCR detects DNA in fragments of *C botulinum* (not active BoNT), and PCR results require confirmation with the mouse bioassay or other validated test.[108] PCR has the advantage of providing results within 24 hours (including serotype of botulism) versus up to 4 days (range 6 to 96 hours) for results from mouse bioassays and 7 to 10 days for cultures. Endopep-MS has a high sensitivity and specificity for detecting botulinum toxin, but generally is used as a secondary assay (ie, if mouse bioassay is negative or cannot be done due to inadequate sample). Other tests in various stages of development for detecting botulinum toxin (ie, lateral flow, endopeptidases, enzyme-linked immunosorbent assays, and electrochemiluminesence assays) have been developed.[108]

Foodborne Botulism

Toxin assays of specimens from foodborne botulism cases from 1975 to 1988 showed the presence of toxin in specimens from various sites as follows: sera, 53% (126/240); stool, 23% (65/288); and gastric aspirate, 5% (3/63). Specimens were more likely to be positive if obtained soon after toxin ingestion. Toxin assays of sera were positive in more than 60% of specimens obtained within 2 days after toxin ingestion, in 44% of specimens obtained within 3 days of toxin ingestion, but in only 23% of specimens obtained at day 4 or later.[98] Toxin assays of sera were more likely to be positive in intoxications from toxin serotype A than from toxin serotypes B and E. Toxin assays of the stool were positive in 50% of specimens obtained within 1 day following toxin ingestion, in 39% of specimens obtained within 3 days of ingestion, but in less than 20% of specimens obtained at day 5 or later.[99]

Stool and gastric aspirate cultures for *C botulinum* resulted in a higher yield of diagnosis than toxin assays.[99] Gastric aspirates were positive in 45% of specimens (35/78). Nearly 80% of stool cultures were positive at day 2 postingestion of toxin, with nearly 40% of specimens remaining positive at 7 to 9 days after ingestion. However, in this cohort of patients, laboratory confirmation of botulism could not be obtained in 32% of patients, which reflects the insensitivity of the diagnostic testing, especially when specimens are obtained more than 3 days after toxin ingestion. In these patients, the diagnosis must be based on clinical history, physical examination, electromyography results, epidemiological history (including food consumption), and tests on ingested food samples from epidemiologically linked food.

Inhalation-acquired Botulism

Laboratory confirmation of botulism acquired by inhalation may be difficult, because toxin acquired by inhalational exposure is not generally identifiable in the serum or stool, as in foodborne botulism.[121,122] Although not validated, an enzyme-linked immunosorbent assay or PCR test has been reported to detect botulinum toxin (using nasal mucosal swabs) from the nares for up to 24 hours after exposure.[122,123] Antibody titers also have limited use in the diagnosis of botulism, because individuals may not develop an antibody response to the small quantity of toxin protein required to cause symptoms. Additionally, cultures of *C botulinum* are not helpful for definitive confirmation of inhalation of toxin preparations that do not contain spores of the organism.

Other Forms of Botulism

In wound botulism, serum toxin assays have been reported in one US cohort to be positive in 95% of cases associated with injecting drug use (mainly subcutaneous injection of black tar heroin) and in 83% of cases not associated with injecting drug use, but reported in other cohorts to be lower (range 38% to 68%).[108] Toxin assays from the wound were reported positive in a third of cases, and *C botulinum* was cultured from the wound in 65% of cases (61% of cases in injecting drug use-associated and 83% of noninjecting drug use-associated botulism).[108] Laboratory confirmation of infant botulism is often by toxin assay and culture of stool (positive in nearly all cases); toxin in the serum is less sensitive (only 13% [9/67] positive in one US cohort).[108,124] Laboratory confirmation of iatrogenic botulism associated with injection of therapeutic preparations of botulinum toxin is by toxin detection in serum.[125]

TREATMENT

The current recommended treatment for botulism, although limited, includes antitoxin therapy and supportive care as needed, including mechanical ventilation. Because respiratory failure may begin suddenly, individuals with suspected botulism should be closely monitored, with frequent assessment of the vital capacity and maximal inspiratory force.[126] If ingestion of the implicated food has been recent, removal of unabsorbed toxins may be hastened with cathartic agents or enemas, provided ileus is not present. Decrease in gastric motility may require parenteral nutritional support. In wound botulism, antibiotic therapy and surgical debridement are recommended to remove the source of toxin production. Wound manipulation preferably should be done after antitoxin therapy administration because it may result in release of toxin into the bloodstream.[105] Aminoglycosides, clindamycin, and magnesium containing medications should be avoided, if possible, as they may potentiate neuromuscular blockade.[127–129]

Antitoxin

Mortality from foodborne botulism before 1950 was approximately 60%,[51] and has been reduced to less than 10% by the use of antitoxin therapy coupled with supportive care (often mechanical ventilation).[31,101] As botulism antitoxins can only neutralize circulating antitoxin and have no effect on toxin already bound to nerve terminals (antitoxins do not reverse paralysis), antitoxin therapy should be administered as soon as possible.[31,101] Early antitoxin administration has been associated with a decrease in the duration of illness, number of days of mechanical ventilation, requirement for mechanical ventilation, and duration of hospitalization.[130] Early treatment, especially within 24 hours, is most effective in preventing paralysis progression. Therefore, it is recommended that antitoxin treatment commence with clinical suspicion, before the availability of definitive laboratory test results, and especially when a case is epidemiologically linked to a botulism outbreak.[122,120] One retrospective analysis of 134 cases demonstrated a 10% mortality rate in patients who received antitoxin within 24 hours of symptom onset versus 15% among those who received late treatment.[131]

Because antitoxin cannot neutralize toxin once it has bound to the nerve receptors, the antitoxin cannot reverse paralysis; it can only prevent paralysis progression. Symptoms may often progress for 12 to 24 hours after antitoxin administration before an effect is observed.[132] Only one dose of antitoxin treatment is usually required for treatment of botulism. However, a second or subsequent doses may be required if symptoms should continue to progress or relapse, as may occur after exposure to extremely high toxin doses, incomplete wound debridement in wound botulism, or persistent colonization in adult intestinal botulism. In such cases, a serum toxin assay is recommended 24 hours after antitoxin administration.[108] Antitoxin levels in four botulism patients given trivalent equine botulism antitoxin had previously demonstrated peak serum levels of antitoxin 10 to 1,000 times higher than the amount required to neutralize serum toxin levels.[133]

In 2010, equine antitoxins available at CDC were replaced with an investigational new drug (IND) despeciated equine heptavalent (A-G) antitoxin product (Cangene Corporation, Winnepeg, Canada) then known as HBAT. It was subsequently FDA approved in 2013 and renamed as BAT (Emergent Biosolutions, Gaithersburg, MD).[134,135] Despeciated equine antitoxins are made by cleaving the Fc fragments from the horse immunoglobulin G molecules, resulting in only $F(ab')_2$ and Fab' fragments that contain less than 2% horse protein. The decrease in the amount of horse protein may potentially reduce the risk of serum sickness and hypersensitivity reactions as observed with nondespeciated equine botulinum antitoxin products (1% risk with a single vial and 9% risk with 2 to 4 vials).[136] Symptoms in clinical trials in 16 healthy subjects were headache (9%), pruritus (5%), nausea (5%), and urticaria (5%). Safety data from 213/216 adult and 13/15 pediatric subjects who received BAT for suspected or confirmed botulism (5 subjects receiving 2 doses) noted 10% of subjects reported adverse reactions.[137] Most common reactions reported were fever (4%), rash (2%), chills (1%), nausea (1%), and edema (1%). No subject experienced anaphylaxis, but one subject experienced mild serum sickness and one subject experienced hemodynamic instability characterized by bradycardia, tachycardia, and asystole during BAT administration. Rebound intoxication was observed in a case of adult intestinal botulism resulting from toxin serotype F and occurred 10 to 12 days after receiving the BAT.[2] The rebound was attributed to the more rapid clearance of BAT from the circulation due to BAT's shorter estimated serum half-life, and also due to intestinal colonization and toxin production from *Clostridia*.[2,108]

BAT is the only botulism antitoxin available for noninfant patients. In emergencies it may be obtained from the CDC (contact the state or county health department or, alternatively, the CDC Emergency Operations Center at 770-488-7100 or 800-CDC-INFO [800-232-4636], or http://www.cdc.gov/phpr).[134,135]

For treatment of infants with botulism resulting from toxin serotypes A and B, an FDA-approved bivalent (A/B) human botulism immune globulin product (BabyBIG/BIG-IV),[138] is available at the California Department of Health.[1,114,138] BabyBIG was derived from pooled plasma of adults immunized with an investigational botulinum toxoid. Because it is derived from humans, BabyBIG does not have the high risk of anaphylaxis observed with equine products, nor the risk of lifelong hypersensitivity to equine antigens. A placebo-controlled trial with BabyBIG in treating infant botulism (associated with a mortality less than 2% even with supportive care without antitoxin) demonstrated efficacy by decreased duration of hospital stay, intensive care unit stay, mechanical ventilation, tube feedings, and a $55,000 cost savings per case.[114] A single infusion of BabyBIG has been estimated to neutralize toxin for up to 5 months, based on its prolonged half-life of approximately 28 days.[139] BabyBIG may interfere with the response to live viral vaccines if given shortly before or within 5 months after BabyBIG administration. When botulism in infants is not due to toxin serotypes A or B (or if BabyBIG is not available), HBAT may be considered, as it has been successfully administered to one infant in the United States.[140] Also, a retrospective review of 31 cases of infant botulism in Argentina treated with equine botulinum antitoxin was well tolerated (no serious hypersensitivity reactions) and was associated with a reduction in hospital stay and tube feeding by 24 days and mechanical ventilation by 11 days and a 47% decrease in sepsis.[141]

Animal studies with a heptavalent despeciated antitoxin (IND product developed at the US Army Medical Research Institute of Infectious Diseases that was a basis for BAT development) demonstrated efficacy in preventing and treating botulism in both mice and nonhuman primates against aerosol toxin challenge.[97,134,142] The $F(ab')_2$ heptavalent, despeciated equine antitoxin toxin (known as Hfab-BAT) given to asymptomatic mice within a few hours after aerosol challenge with approximately 10 LD_{50} of serotype A, was protective, even with a dose as low as one-tenth of one human dose. This dose resulted in low levels of antitoxin titers, 0.02 IU/mL or lower.[97] The product was also protective against aerosol challenge to toxin serotype A at a dose of approximately 2,000 mouse intraperitoneal LD_{50}/kg, when given to nonhuman primates immediately before exposure (protection in 5/5 animals), and when given 48 hours after inhalational exposure (protective in 3/5 monkeys).[97]

However, if antitoxin was given at the onset of respiratory failure, the Hfab-BAT product was not protective in the mouse model against aerosol exposure or intraperitoneal exposure, even with a dose that was 3-fold greater than the recommended human-equivalent dose. The ineffectiveness of delayed antitoxin administration in mice may be because the majority of toxin is no longer present in the circulation at the time of the antitoxin administration (ie, it is already bound to nerve terminals). Respiratory failure in mice occurred within 1 to 3 hours, and death occurred within 2.8 to 11 hours postexposure, which is earlier than observed in humans and nonhuman primates whose death generally does not occur until 2 to 3 days postexposure. In one review of human foodborne botulism, shortness of breath at presentation was also identified as a poor prognostic factor for survival, even with antitoxin therapy; it was noted in 94% (50 of 55) of the deaths.[72] The Hfab-BAT and the HE-BAT despeciated equine antitoxin (also developed at the US Army Medical Research Institute of Infectious Diseases) are no longer available as IND products, and they have been replaced with the FDA-approved BAT despeciated antitoxin product.

In a successful Phase I clinical trial, a product made from three IgG1 monoclonal antibodies (Xoma 3AB) that target different regions of botulinum neurotoxin A and were engineered to neutralize toxin serotype subclasses A1, A2, A3, and A4 was well tolerated when given as a single intravenous injection infused over 1 hour.[143] The product has demonstrated a reduced mortality in mice when given prior to or up to 23 hours after toxin exposure. Although all three monoclonal antibodies were detected for a minimum of 4 weeks after infusion, the protective level of the monoclonal antibodies in humans is currently unknown. Further testing of this product may provide an antitoxin therapy for botulism that offers potential advantages of (1) a longer serum half-life, (2) decreased risk of allergic reactions (contains no residual equine proteins), and (3) mass production potential.[144,145]

Clinically Relevant Signs of Bioterrorist Attack

The first evidence of a bioterrorist attack with botulinum toxin would likely be reports from hospitals and urgent care medical facilities as they begin to receive victims with symptoms suggestive of botulism. Because antitoxin therapy given early has a greater beneficial effect, the initial diagnosis of botulism is based on clinical presentation with epidemiological associations, with subsequent confirmation by laboratory findings.[34] Neurological signs and symptoms resulting from a toxin-induced blockade of neurotransmission at voluntary motor and cholinergic junctions dominate the clinical manifestation of botulism.[98,146,147] A diagnosis of botulism is suggested in individuals presenting with an acute onset of cranial nerve weakness (ie, dip-

lopia, ptosis, dysphonia, dysphagia, and dysarthria). In mild cases, no further symptoms may develop. In more severe cases, individuals may progress and develop descending symmetrical weakness and flaccid paralysis. Because mechanical ventilation may be required for individuals with respiratory failure resulting from paralysis of the respiratory muscles, hospital bioterrorism plans should include contingency plans for additional ventilatory and intensive care unit support for mass intoxication. Antitoxin therapy is indicated in cases of suspected botulism to inactivate and clear toxin from the circulatory system before it can enter peripheral cholinergic nerve cells.

An outbreak of botulism in 2004 illustrates the vulnerability of readily accessible bulk botulinum toxin. Four cases of botulism resulted from the use of toxin serotype A for cosmetic purposes. A vial of raw bulk botulinum toxin (a non-FDA approved formulation) containing between 20,000 and 10 million units of botulinum toxin (a vial of FDA-approved BOTOX [Allergan, Inc, Irvine, CA] contains only 100 units of toxin) was used by an unlicensed physician for cosmetic injections into three patients and himself.[148,149] The four individuals were subsequently admitted to medical facilities with symptoms of botulism and faced a long-term recovery.[150] One of these patients was iatrogenically injected with as much as 8 million mLD_{50} (2,857 times the human lethal dose by injection) and has survived with intensive medical care, including long-term mechanical ventilation, although with chronic sequelae.[106,150] This patient began improving 4 weeks after the event, and achieved a remarkable recovery 14 weeks from injection.[107]

Preexposure and Postexposure Prophylaxis

Although passive antitoxin prophylaxis has been effective in protecting laboratory animals from toxin exposure, the limited availability and short-lived protection of antitoxin preparations make preexposure or postexposure prophylaxis with these agents impractical for large numbers of persons.[121,151] Administration of equine antitoxin is not recommended for preexposure prophylaxis because of the risk of anaphylaxis from the foreign equine proteins, particularly with repeated doses. These products are not generally recommended for use in asymptomatic persons. In asymptomatic persons with known exposure to botulinum toxin, the risk of anaphylaxis from the equine antitoxin must be weighed against the risk of disease from botulinum toxin. However, botulinum immune globulin is most effective when administered within 24 hours of a high dose aerosol exposure to botulinum toxin.

No FDA-approved vaccines exist to prevent botulism. Of historical note, a bivalent botulinum toxoid (serotypes A and B) had been given to at-risk laboratory workers in the US offensive biological warfare program at Fort Detrick beginning in 1945.[152] Between 1945 and 1969, 50 accidental exposures to botulinum toxins (24 percutaneous, 22 aerosol, and 4 ingestion) were reported, but no cases of laboratory-acquired botulism occurred, possibly because of the toxoid immunizations. An IND product, the pentavalent botulinum toxoid (PBT) against botulinum toxin serotypes A through E, had been used since 1959 for persons at risk for botulism (ie, laboratory workers)[152–154] but is no longer available as an investigational product on protocol through the CDC. In 2012, due to declining immunogenicity and increased local reactogenicity observed with the requirement for annual booster doses to maintain immunity,[155–157] the CDC discontinued its sponsorship of IND that provided PBT to at-risk laboratory workers.[158–160] The declining immunogenicity of the PBT was not unexpected, given that the PBT was manufactured more than 30 years earlier. PBT is a toxoid (toxin that has been inactivated) derived from formalin-inactivated, partially purified toxin serotypes A, B, C, D, and E, which was developed by the Department of Defense at Fort Detrick and originally manufactured by Parke-Davis and Company (Detroit, MI). PBT was found to be protective in animal models against challenge with botulinum toxin serotypes A through E,[161] including protection in nonhuman primates against aerosol challenge to toxin serotype A.[162]

PBT was originally given as a primary series of three subcutaneous injections (0.5 mL at 0, 2, and 12 weeks), a booster dose at 12 months, and annual booster doses thereafter.[163] PBT was administered to thousands of at-risk persons, and clinical experience has shown the toxoid to be safe and immunogenic. The vaccine has mainly been used for laboratory workers who work directly with botulinum toxin. Approximately 8,000 service members also received the toxoid between January 23 and February 28, 1991, as part of the US force deployed to the Persian Gulf War.[164] The main adverse event was local reactions. Adverse events passively reported to the CDC between 1970 and 2002 for more than 20,000 vaccinations included moderate local reactions (edema or induration between 30 mm to 120 mm) in 7% of vaccinees, and severe local reactions (reaction size >120 mm, marked limitation of arm movement, or marked axillary node tenderness) in less than 1%.[158] To allow recent vaccinees to complete the primary series of PBT, the investigational new drug protocol remained in effect through May 31, 2012.[158]

New Vaccine Research

Future vaccine candidates for FDA approval will probably not include formalin-inactivated toxoids similar to the formalin-inactivated PBT for several reasons.[165,166] Production of formalin-inactivated toxoids requires partially purified culture supernatants containing botulinum toxin to be treated with formaldehyde, which must be performed by highly trained staff using a dedicated high-containment laboratory space.[167] Furthermore, the relative impurity of the toxoid (PBT contained only 10% neurotoxoid and 90% is irrelevant material) likely contributed significantly to the need for multiple injections to achieve and sustain protective titers, as well as increased local reactogenicity associated with multiple injections.

The use of pure and concentrated antigen in recombinant vaccines offers the advantages of increased immunogenicity and a decrease in reactogenicity (local reactions at the injection site) over formalin-inactivated toxoids.[168] Recombinant techniques use an immunogenic toxin fragment, which does not have the capability of blocking cholinergic neurotransmitters. Both *Escherichia coli* and yeast expression systems have been used in recombinant fragment production, primarily the carboxy-terminal fragment (Hc) of the toxin's heavy chain. Vaccine candidates using recombinant fragments of botulinum toxins against serotypes A, B, C, E, and F were protective in mice.[169–177] A vaccine recombinant candidate for serotype A was protective in mice against intraperitoneal challenge and produced immunity levels similar to that attained with PBT, but with an increase in safety and decrease in cost per dose.[167] Recombinant vaccines given by inhalational route are also being investigated.[178,179]

The only botulinum vaccine candidate currently in advanced development is a bivalent recombinant botulinum vaccine (rBV A/B [*P pastoris*] for toxin serotype A and B) developed by the US Army Medical Research Institute of Infectious Diseases for protection against botulinum toxin serotype A (subtype 1) and botulinum toxin serotype B (subtype B1).[180] Animal studies have demonstrated protection against toxin challenge by aerosol and intramuscular challenge with the two toxin serotypes.[181] The safety data and immunological responses from Phase 1 and Phase 2 clinical trials and animal data support the continued investigation of the rBV A/B for FDA vaccine licensure.[179,181–184]

Another vaccine candidate evaluated involved the insertion of a synthetic carboxy-terminal fragment (Hc) gene of the heavy chain of toxin serotype A into the vector system of the Venezuelan equine encephalitis virus.[173] This vaccine induced a strong antibody response in the mouse model and remained protective in mice against intraperitoneal challenge at 12 months. However, the presence of BoNT variants as subtypes or chimeras challenges the development of any comprehensive "pan-botulinum neurotoxin" vaccines and therapies.[185] BoNT subtypes are natural variants of the prototype BoNT serotype that vary in primary amino acid sequence from 3% to 26% depending on the serotype, whereas BoNT chimeras are natural variants that appear to have derived from recombinant events between two BoNT serotypes.[186] BoNT subtypes have unique activities relative to the prototypical BoNT serotype.[186] One novel approach to vaccine design uses structure-based knowledge to produce a single molecule containing the immunodominant epitopes of multiple, antigenic distinct variants to develop a meningococcal pan-vaccine.[187] A similar research effort may eventually allow the development of a similar pan-vaccine strategy for all seven variants of botulinum neurotoxins.[188]

New Therapeutic Drug Research

In addition to the vaccine research for prophylaxis, efforts are underway to find postexposure, pharmacologic treatments. The development of low molecular weight inhibitors is related to work that was designed to pharmacologically "deduce commonalities" among the serotypes.[189] Different molecular targets for inhibitor candidates include those that prevent toxin binding to nerve terminals and internalization or those that inhibit the neurotoxin's proteolytic activity. One limitation for this approach to be useful is that when the neurotoxin molecule is internalized within the presynaptic endings, the neurotoxin is—as with neutralizing antibodies—no longer susceptible to circulating inhibitors. Thus, effective therapeutic inhibitors must also be internally delivered to nerve termini without creating additional adverse central effects. Another problem concerns the diversity of the substrate binding sites (exosites)[190] and active site structures among the serotypes.[191] This diversity implies that a single inhibitor will not antagonize the substrate binding and subsequent proteolytic activity of all the neurotoxin serotypes.

Active site inhibitor candidates have been extensively reviewed[192,193] with new candidates appearing frequently in the literature.[194–197] Within the BotDB resource (http://botdb.abcc.ncifcrf.gov),[198] the BotDBI section[199] has information on more than 60 inhibitor candidates that lists peptides, synthetic and natural compounds, and antibodies.[200]

A steady rise over the past 30 years has occurred in the clinical/therapeutic uses of this neurotoxin for vari-

ous disorders. The FDA-approved uses include treatment of blepharospasm, strabismus, cervical dystonia, upper limb spasticity, axillary hyperhidrosis, detrusor overactivity associated with a neurologic condition, and chronic migraine.[200] Other potential uses are still under investigation including those for wound healing,[201] treatment of cerebral palsy,[202,203] treatment of lower urinary tract disorders,[204,205] controlling multiple sclerosis spasticity,[206] and various modalities of pain management.[207–209] Because of these clinical conditions, very small doses of the neurotoxin may be used for a measurable beneficial effect. One novel clinical application involves the neurotoxin-induced reduction of hyperhidrosis.[210]

SUMMARY

The neurotoxins produced by *Clostridia* species are among the most potent toxins known. Botulinum toxin has been studied and developed as a biological weapon by many countries, and it should be considered as a bioterrorism threat agent. A mass casualty event caused by botulinum toxin, which has been depicted by a mathematical model, has the potential to cause great harm. Botulism is most commonly caused by neurotoxin types A, B, and E, is a neuroparalytic disease, and often fatal if untreated. Paralysis from botulism can be long-lasting, with concomitant and demanding supportive care requirements. Clinicians should be able to recognize the early signs and symptoms of botulinum intoxication, as early initiation of antitoxin therapy has been associated with decreased duration of mechanical ventilator support and days spent in intensive care, and increased survival. Antitoxin therapy should be initiated based on clinical presentation consistent with botulism and epidemiological history, as results of laboratory confirmatory assays may not be available for days.[120]

For infants with botulism resulting from toxin serotypes A and B, an FDA-approved human antitoxin known as BabyBIG is available at the California Department of Public Health.[143] An FDA-approved despeciated equine heptavalent antitoxin product known as BAT (serotypes A-G) is the only antitoxin for adults (and for infants if BabyBIG is not available or botulism not due to serotypes A or B), and is available through the CDC.[120] No FDA-approved vaccine for botulism currently exists. The IND PBT product is no longer available for protection of at-risk laboratory workers because of declining immunogenicity and increased local reactogenicity associated with required annual booster doses.[122] Future vaccine research may lead to a new class of recombinant vaccines to protect against botulism while pharmacologic approaches may lead to viable postexposure drug treatments.

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Chapter 15

CLOSTRIDIUM PERFRINGENS EPSILON TOXIN

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INTRODUCTION

Clostridium perfringens is a gram-positive, spore-forming anaerobe commonly found throughout nature (ie, soil, water, gastrointestinal tracts of humans and animals, etc). This bacillus is one of the most "toxic" bacteria described to date, producing at least seventeen different "major" and "minor" protein toxins.[1,2] Other pathogenic species of *Clostridium* synthesize the most potent protein toxins known, such as tetanus and botulinum neurotoxins. Unlike a number of other bacterial pathogens (ie, *Listeria*, *Rickettsia*, *Salmonella*, *Shigella*, and *Yersinia* species), current understanding of *C perfringens* pathogenesis during various diseases does not include invasion, and subsequent replication, in eukaryotic cells.

C perfringens was first isolated in 1892 by William Welch and George Nuttall at Johns Hopkins University in Baltimore following an autopsy of a cancer patient. Of note was a rather profuse, unusually explosive formation of gas bubbles within the cadaver's blood vessels and organs only 8 hours after death. Gas is a common byproduct of anaerobic growth by clostridial species, explaining the term "gas gangrene" during severe myonecrosis induced by *C perfringens*. Over time and throughout the literature, *C perfringens* has also been known as *Bacillus aerogenes capsulatus*, *Bacillus welchii*, and *Clostridium welchii*. Many ill-effects induced by *C perfringens* in humans and animals are linked to protein toxins. Below are succinct descriptions of the classically defined major (typing) toxins with a particular emphasis on epsilon, which has been targeted recently as a select agent by various agencies within the United States and other countries.

HISTORY

Protein toxins are considered important virulence factors for *C perfringens*, and have thus received much attention by various laboratories throughout the world. For many bacterial pathogens, toxins possessing diverse modes of action play critical roles in survival that include nutrient gathering and thwarting the host's immune system. There are two primary modes of action described for the four major (typing) toxins produced by *C perfringens*: 1) increasing permeability of cell membranes (ie, alpha, beta, and epsilon toxins) resulting in ion imbalances and general leakiness; and 2) destroying the actin cytoskeleton (ie, iota toxin).[2] In either scenario, the end result elicited by any of these toxins is the same: cell dysfunction and death. Multiple studies by many groups reveal that *C perfringens* possesses highly evolved tactics, involving offensive (ie, secreted protein toxins plus enzymes) and defensive (ie, protein toxins plus spores) tools for surviving, and then thriving, in harshly diverse environments. *C perfringens* consists of five toxin types: A, B, C, D, and E (Table 15-1), based on the production of one or more protein toxins.[1,2]

Each of these toxins is lethal, dermonecrotic, and associated with a wide range of diseases that include a rapid, life-threatening myonecrosis (gas gangrene) and various enteric illnesses in both animals as well as humans (Table 15-2). Historically, for diagnostic purposes these typing toxins would be neutralized in the laboratory by type-specific antisera in mouse-lethal and guinea-pig dermonecrotic assays. The toxin source would consist of culture filtrate from *C perfringens* isolated from a patient.[3] Today, rapid genetic methods involving multiplex polymerase chain reactions are more commonly used by diagnostic laboratories around the world for typing *C perfringens*.[4,5] This technique, although rapid and accurate, suggests the presence of a toxin gene but indicates neither production nor relative quantities of a biologically-active protein. Rapid quantitation of epsilon toxin protein in complex matrices (eg, milk and serum) is also possible using a novel, mass spectrometry technique; however, this does not determine whether the toxin is biologically active.[6]

Unlike the other typing toxins, alpha is, by definition, produced by all *C perfringens* and has played a significant role in military casualties over time. Type A strains are most commonly found in the environment and cause gas gangrene.[1,7–11] Alpha toxin facilitates gas gangrene due to *C perfringens* infection, an omnipresent threat to soldiers wounded on the battlefield.[7,8] Deep penetrative wounds, soiled by dirt that contains

TABLE 15-1

TOXIN TYPES OF *CLOSTRIDIUM PERFRINGENS*

Major (typing) Toxins	Toxin Type				
	A	B	C	D	E
Alpha	√	√	√	√	√
Beta		√	√		
Epsilon		√		√	
Iota					√

TABLE 15-2

***CLOSTRIDIUM PERFRINGENS* TOXIN TYPES AND DISEASES**

Toxin Type	Disease/Intoxication
A	Myonecrosis (gas gangrene), necrotic enteritis of fowl and piglets, human food poisoning, antibiotic-associated diarrhea
B	Dysentery in lambs; hemorrhagic enteritis in calves, foals, and sheep
C	Necrotizing enteritis in humans (known as pigbel, darmbrand, or fire-belly), pigs, calves, goats, and foals; enterotoxemia in sheep (struck)
D	Enterotoxemia in lambs (pulpy kidney or overeating disease) and calves; enterocolitis in goats and cattle
E	Calf and lamb enterotoxemia

various clostridial species (especially *C perfringens*), are often to blame for quickly advancing disease in the buttocks, thigh, shoulder, upper extremity, and leg (in order of decreasing prevalence).[7,10,11] The fatality rate from gas gangrene was 50% during World War II; it was especially high when fighting occurred in cultivated land (commonly fertilized with animal feces), as opposed to desert (eg, Tunisia).[10,11] The threat of gangrene from *C perfringens* or other clostridial species[11] due to wound contamination in the field or nonsterile operating conditions was particularly prevalent before 1900 and resulted in many amputations and deaths that can be avoided in modern times. If administered promptly after disease onset, medical countermeasures, such as extensive surgical debridement, various antibiotics (eg, beta-lactams, clindamycin, metronidazole), and hyperbaric oxygen provide effective treatment for most cases of *C perfringens*-induced gangrene. Antitoxin (historically, polyclonal antibodies of equine origin) administration is also another possible therapy that targets alpha toxin and mitigates myonecrosis.[7,10,12] Vaccine studies from various groups using the carboxy-terminal (cell binding) domain of alpha toxin show prophylactic protection against either toxin-induced lethality or bacterial challenge in a mouse gangrene model.[13,14]

Biochemically, alpha toxin is a zinc-containing phospholipase C (43 kDa) composed of two structural domains that destroys eukaryotic cell membranes.[15,16] The amino-terminal domain contains a catalytic site and ganglioside (GM1a) binding motif, the latter being similar to that found on another clostridial toxin studied by the biodefense community: *Clostridium botulinum* neurotoxin.[17] Interaction of GM1a by alpha toxin promotes clustering and activation of tyrosine kinase A involved in signal transduction.[17] The carboxy-terminal domain of alpha toxin binds to phospholipids on cell membranes.

In comparison to the alpha toxin and due to recent national and international biodefense concerns, *C perfringens* epsilon toxin has received much more government attention (ie, funding and regulated oversight) over the past 15 years as a potential agent used in biowarfare and bioterrorism.[18] Epsilon is the most potent of all *C perfringens* toxins as determined by a very low LD_{50} (toxin amount necessary to kill 50% of the subject population; murine intravenous assay), ranking behind only the *C botulinum* and *C tetani* neurotoxins among all clostridial toxins. In the very recent past, the Centers for Disease Control and Prevention placed epsilon toxin on the Category B list of select agents, along with bacterial diseases (eg, brucellosis, glanders, and typhus) plus other protein toxins (eg, ricin and staphylococcal enterotoxin B). Additionally, epsilon toxin represented a potential agroterrorism threat and was deemed a select agent by the United States Department of Agriculture. However, when the select agents list was modified in December 2012, *C perfringens* epsilon toxin was removed.[19]

In France, but not throughout Europe, epsilon toxin is still classified as a potential biological weapon and requires special authorization from a federal agency (Agence Nationale de Securite du Medicament) before being approved for laboratory work. There are varying opinions around the world regarding *C perfringens* epsilon toxin, its potential nefarious applications, and imposed level of government regulations. Such nonconsensus among allies affects sustained funding and effective collaborations between investigators, laboratories, and nations.

DESCRIPTION OF THE EPSILON TOXIN

Natural Occurrence

The epsilon toxin is naturally produced by types B and D *C perfringens* that are involved in animal (cattle, goat, and sheep) enterotoxemias that often prove widespread, rapidly fatal, and economically damaging for the agriculture industry.[1,20] Although *C perfringens* is considered normal intestinal flora in ruminants, types B and especially D can cause severe, life-threatening illness in a "naïve" digestive system shortly after

birth, or following a diet change involving higher carbohydrate levels (particularly starch) among older animals. If there is little microbial competition within the gastrointestinal tract or an overly nutrient-rich diet is suddenly consumed, resident *C perfringens* types B and D can rapidly proliferate in the intestines and concomitantly produce life-threatening levels of toxins that include epsilon. Those who study *C perfringens* and disease naturally associate the epsilon toxin with veterinary issues. In fact, neither the epsilon toxin nor *C perfringens* types B and D infections are commonly linked to human disease, which may make the toxin ideal for nefarious use as a biological weapon against humans: a bioterrorist event employing epsilon toxin against humans could be very difficult to diagnose and treat because there is no "natural" precedent and classically trained physicians do not anticipate human illness linked to *C perfringens* epsilon toxin.

Chemical and Physical Properties

C perfringens epsilon toxin is a plasmid-borne, 311 amino acid (32.9 kDa) protein secreted as a protoxin, activated subsequently by extracellular proteases (trypsin and chymotrypsin) that remove amino-terminal (14 amino acids) and carboxy-terminal (29 amino acids) peptides.[20] The nascent protoxin contains a typical leader sequence (32 amino-terminal residues) that normally facilitates protein secretion from the bacterium into the environment. The toxin is resistant to inactivation by serine-type proteases commonly found throughout nature, including those in the gastrointestinal tracts of various mammals.

The crystal structure of epsilon toxin (Figure 15-1) reveals an elongated beta-sheet (100 Å × 20 Å × 20 Å) containing three domains, which shares conformation with other bacterial (aerolysin family) pore-forming toxins, including *Aeromonas hydrophila* aerolysin, *C perfringens* enterotoxin, and *Clostridium septicum* alpha toxin.[21,22] The putative roles for each domain of epsilon toxin include receptor binding (domain I: amino terminus), channel formation (domain II: central region), and monomer-monomer interaction (domain III: carboxy terminus).[2,21,23] Loss of a carboxy-terminal peptide from epsilon toxin seems primarily responsible for monomer-monomer interactions and subsequent homoheptamer formation.[23]

Proteolysis is a common method of activating many bacterial toxins, and for epsilon this process induces conformational changes that facilitate homooligomerization on the external surface of a eukaryotic cell. Essentially, proteolytic activation causes "protein priming" that enables the toxin to quickly act after binding to diverse target cells of neuronal, renal, and endothelial origins (described in detail below). Additionally, proteolysis of the amino- and carboxy-termini on the epsilon protoxin leads to a more acidic isoelectric point (5.4 versus 8.0), which may play a role in receptor interactions.[20,24] For enteric-produced toxins requiring proteolysis, the proteases synthesized by resident bacteria (including *C perfringens* lambda toxin)[25] and host[23] are bountiful. Recent evidence suggests that epsilon toxin can be activated intracellularly (in select strains), remains in *C perfringens* until stationary or death phase, and is finally released into the environment following autolysis.[26] This unique protease and a further understanding of its novel activation mode in clostridia (and possibly in other secreted toxin-producing pathogens) remains elusive.

Mechanism of Action

The mode of action for epsilon toxin involves pore formation in eukaryotic cell membranes facilitated by detergent-resistant membrane fractions, also known

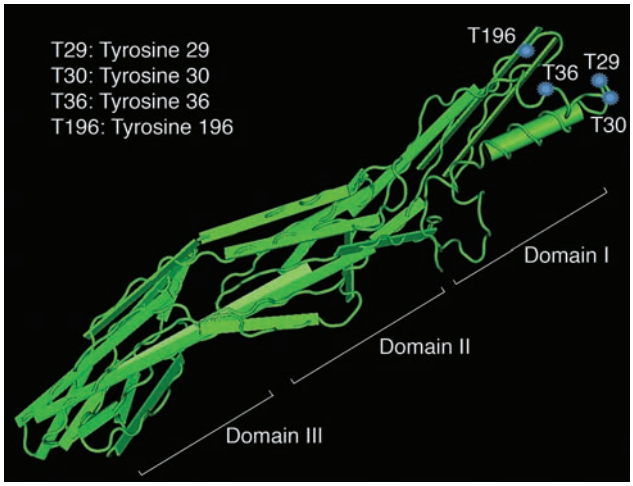


Figure 15-1. Crystal structure of *Clostridium perfringens* epsilon toxin. Three domains exist and are putatively involved in (I) receptor binding (amino terminus), (II) channel formation, and (III) monomer-monomer interactions (carboxy terminus). Designated amino acids (tyrosines 29, 30, 36, and 196) facilitate receptor (hepatitis A virus cellular receptor 1) binding, and when individually replaced with glutamic acid, yield nontoxic variants of epsilon toxin.
Data sources: (1) Cole AR, Gibert M, Popoff MR, Moss DS, Titball RW, Basak AK. *Clostridium perfringens* epsilon-toxin shows structural similarity to the pore-forming toxin aerolysin. *Nat Struct Mol Biol*. 2004;11:797–798. (2) Ivie SE, McClain MS. Identification of amino acids important for binding of *Clostridium perfringens* epsilon toxin to host cells and to HAVCR1. *Biochemistry*. 2012;51:7588–7595. (3) Madej T, Addess KJ, Fong JH, et al. MMDB: 3D structures and macromolecular interactions. *Nucleic Acids Res*. 2012;40:D461–464.

as lipid rafts, that concentrate toxin monomers into homoheptamers.[27,28] These cholesterol-rich membrane domains play important roles in many diseases elicited by bacteria, associated toxins, and viruses.[29] Furthermore, caveolins 1 and 2 found in these domains are bound directly by epsilon toxin and necessary for toxin oligomerization.[30] Epsilon toxin oligomers, critical for biological activity:

- form within 15 minutes at 37°C on Madin-Darby canine kidney cells (MDCK);
- are more stable at 37°C versus 4°C;
- promote potassium efflux; and
- are internalized from the cell surface, forming vacuoles in the late endosomes and lysosomes.[31,32]

Sialidases, also known as neuraminidases, are produced by *C perfringens* and enhance binding of the bacterium as well as epsilon toxicity to select cultured cells.[33] For example, in the presence of bacterial-derived sialidase, there is increased adherence of *C perfringens* to a human colorectal epithelial line (Caco-2), but not to MDCK or Vero (monkey kidney) cells. Contact between the bacterium and certain cell lines like Caco-2 leads to gene upregulation and increased activity for *C perfringens*-derived sialidase. Increased toxin binding to target cells following sialidase activity has been described previously for another enteric toxin-producing pathogen, *Vibrio cholerae*.[34]

In concert with disrupted cell membranes facilitating free passage of 1 kDa-sized molecules,[35] secondary effects of epsilon toxin involve cytoskeletal dysfunction that becomes lethal for an intoxicated cell.[36] Additionally, the integrity of a cell monolayer is readily disrupted by epsilon toxin,[27] providing another clue toward understanding subsequent dysfunction of the vascular endothelium, edema, and crossing of the blood-brain barrier.[37] The ill effects induced by epsilon toxin upon the circulatory system are quite substantial, with albumin-sized molecules (~ 65 kDa) subsequently transiting from the blood stream into the brain.[38]

CLINICAL SIGNS AND SYMPTOMS

Although epsilon toxin can be found in the heart, lungs, liver, and stomach following intoxication, it noticeably accumulates in kidneys, causing what veterinarians classically refer to as "pulpy kidney disease."[1,2,20,39–41] Another indicator that kidneys are a primary target of epsilon toxin is that the few susceptible cell lines discovered to date are of kidney descent from dog, mouse, and human.[20] Postmortem results from lambs and mice given epsilon toxin show similar results in the kidneys that include congestion, interstitial hemorrhage, and degenerated epithelium within the proximal tubules. Toxin accumulating in the kidney may represent a natural defense mechanism by the host to prevent lethal concentrations in the brain.[41] During lamb enterotoxemia, glucose excretion into the urine following epsilon toxin exposure may be a result of liver-released glycogen.[42]

The ability of *C perfringens* epsilon toxin to rapidly disrupt the blood-brain barrier, bind neuronal cells, and cause lethality is of obvious concern.[20,37,39] Among neuronal cell populations (sheep), neurons are most susceptible, followed by oligodendrocytes and astrocytes.[43] In the brain, there are signs of swelling, vacuolation, and necrosis. Edema in the rat brain following an intraperitoneal injection of epsilon toxin causes increased levels of aquaporin-4 in astrocytes, which may be the body's attempt to reduce osmotic pressure surrounding sensitive neurons.[44] Aquaporins are membrane proteins that regulate water flow in various cell types and could be a target for therapies against epsilon toxin. Necrosis of the brain following epsilon intoxication is likely due to multiple factors that include reduced blood flow, sustained hypoxic state, and direct toxicity on various cell types.

Clinical signs attributed to epsilon toxin given intravenously to calves, lambs, and young goats occur rapidly (approximately 30 minutes, depending on dose).[45,46] These animals can experience labored breathing, excited or exaggerated movements, intermittent convulsions, loss of consciousness, and ultimately death. Further signs of epsilon intoxication include elevated blood pressure and vascular permeability, lung edema, and brain congestion with edema.[20] Results from another laboratory reveal that an intravenous injection of epsilon toxin (2–4 LD_{50}, in which an LD_{50} is ~ 70 ng/kg) into mice yields seizures within 60 minutes.[47] More naturally, duodenal inoculation of goats with whole culture or supernatant of *C perfringens* type D leads to diarrhea, respiratory distress, and central nervous system dysfunction (ie, recumbency and convulsions).[48] Similar symptoms are also evident in lambs, minus the diarrhea.[49] Furthermore, in ovines (namely lambs) there can be sudden death or acute disease involving neurological manifestations that include struggling, opisthotonos, convulsions, lateral recumbency, and violent paddling. The mode of action for epsilon toxin in vivo seemingly involves ion imbalance, endothelial disruption, and edema.

A vicious cycle is established by *C perfringens* epsilon toxin in the intestinal tract via increased permeability, leading to higher circulating levels of toxin.[43]

Further studies with a wild-type D culture (sheep isolate, CN1020) given intraduodenally to sheep, goats, and mice reveal that a genetic knockout of epsilon toxin does not cause disease compared to controls.[50] Complementing this mutant with the wild-type toxin gene generates the original phenotype that causes epsilon toxin-based disease. In this thorough study, clinical signs of epsilon intoxication in mice included depression, ataxia, circling, and dyspnea. Overall, these results show that the epsilon toxin from a type D strain is critical for disease in these diverse animal models.

Different animal models show that the toxin is quite active when given intravenously or intraduodenally. However, from a biodefense perspective, critical data are lacking in the literature for epsilon toxin and effects following aerosol delivery. It is unclear what happens to nonhuman primates following epsilon intoxication. To establish a basal level of knowledge for further study and develop medical management strategies for humans, it is necessary to carry out and publish nonhuman primate studies.

MEDICAL MANAGEMENT

From a therapeutic perspective, very little has been done with *C perfringens* epsilon toxin, partly because of its natural association with animal (and not human) disease. An effective vaccine against epsilon toxin (described below) is readily available for animal use, thus obviating the need for a therapeutic in susceptible populations. There is no therapeutic agent or vaccine against epsilon toxin approved for human use at this time. Findings from different laboratories and various in vivo and in vitro studies suggest that therapy is possible. Perhaps a proteomics-based approach following epsilon toxin exposure can reveal unique, host-based targets for therapeutic intervention. This approach has recently been taken in mice given epsilon toxin intravenously, with subsequent analysis of select organs (brain plus kidney), plasma, and urine for differentially-expressed proteins.[51] However, there is much more work to be done on this front.

Miyamoto et al[47] show that riluzole, a benzothiazole (234 Da) used for treating human amyotrophic lateral sclerosis by preventing presynaptic glutamate release, can minimize murine seizures induced by epsilon toxin. These results were manifest after riluzole injection (16 mg/kg, intraperitoneal) given 30 minutes before toxin (140 or 280 ng/kg, intravenous), but the drug was evidently not used in subsequent experiments as a therapeutic administered after toxin. Another murine-based study shows that epsilon toxin binds preferentially to the cerebellum, particularly oligodendrocytes plus granule cells.[52] Incubation of primary-cultured granule cells with epsilon toxin causes a rapid increase in intracellular calcium levels and glutamate release. This study provides a brain cell-specific target (and assay) for therapeutic intervention (and screening of potential therapeutic molecules).[52]

Small molecule libraries have also been screened using an MDCK cell assay for therapeutic effects that counter epsilon toxin.[53] Three structurally unique inhibitors were discovered that do not prevent toxin binding or oligomerization on cells, but likely affect pore function or an unidentified cofactor important in epsilon intoxication. Two of these compounds (*N*-cycloalkylbenzamide and furo[2,3-b]quinoline) protected cells when added 10 minutes after toxin exposure, thus providing therapeutic potential in an in-vitro scenario. Such results logically lead to efficacy studies in animals, though none have been published to date.

Another therapeutic approach against epsilon toxin includes dominant-negative inhibitors, which have been successfully employed as experimental therapeutics for other oligomer-forming bacterial toxins produced by *Bacillus anthracis*, *Escherichia coli*, and *Helicobacter pylori*.[54–57] This concept involves a recombinantly modified toxin that is no longer toxic after deleting a peptide region or substituting specific amino acids. Upon integrating a dominant-negative protein into a complex with wild-type toxin monomers in solution or on a cell surface, a nonfunctional toxin oligomer is generated. Two dominant-negative inhibitors for epsilon toxin were created via cysteine substitutions of four amino acids (isoleucine 51 plus alanine 114, and valine 56 plus phenylalanine 118) that constrain the membrane insertion domain. These paired mutations facilitate an intramolecular cystine bond, oligomer dysfunction (decreased heat and detergent stability plus poor prepore-to-pore transition), and ultimately toxin inactivation in vitro.[57] When used in vitro with MDCK cells, these inhibitors dose-dependently inhibit epsilon-induced cytotoxicity at a 1-, 2-, 4-, or 8 (wild-type toxin)-to-1 (dominant-negative protein) mole mixture. Furthermore, dominant-negative molecules like those presented in this study represent potential vaccine candidates worthy of future study.

Additional therapy and prophylaxis studies show that the epsilon protoxin protects mice (ie, delays time to death) when given intravenously before activated toxin. This protective effect presumably occurs via competitive occupation of the cell surface receptor by the protoxin, primarily localized within the brain.[39]

Such data suggest that a receptor-targeted approach for prophylaxis and therapy is possible; Buxton discovered that a formalin toxoid of the protoxin (100 mg/mouse, intravenous) affords protection up to 100 minutes after intravenous exposure to epsilon toxin (0.5 mg/mouse).[58] The readout in this study was extravasation of horseradish peroxidase from the blood stream into the brain. Recent studies by Dorca-Arévalo et al report a similar binding dissociation constant (K_d ~ 4–6 nM) for the epsilon protoxin and activated toxin to MDCK cells.[59] These results evidently depend on plasma membrane integrity plus an unidentified glycoprotein. Existing literature suggests that further work with receptor antagonists as potential therapeutics against epsilon toxin has not been readily pursued by various laboratories.

Knowledge of cell surface receptors for epsilon toxin and intimate molecular interactions can be useful in formulating effective receptor-based therapies. Early studies by Nagahama and Sakurai reveal that the receptor is perhaps a heat-labile sialoglycoprotein; a pretreatment of rat synaptosome membranes with heat (70°–80°C for 10 minutes), neuraminidase, or pronase effectively reduces the binding of epsilon toxin.[60] Furthermore, this same study reveals that a snake presynaptic neurotoxin (beta-bungarotoxin) decreases epsilon toxin binding in a dose-dependent fashion, suggesting a common receptor for these very different toxins. In contrast, the presynaptic neurotoxin produced by *C botulinum* type A had no effect on epsilon toxin binding. It seems that kidney cells and synaptosomes have different receptors for epsilon toxin, as evidenced by varying results following sialidase pretreatment of cells.[33,60]

Recent studies by Ivie et al show that hepatitis A virus cellular receptor 1 (HAVCR1) acts as a receptor, or coreceptor, for epsilon toxin.[61,62] The natural role of HAVCR1 appears linked to regulatory T cells and maintaining immunological balance. This class I, integral-membrane glycoprotein contains multiple isoforms varying within a mucin-like domain that possesses multiple glycosylation sites. Domain I tyrosines (29, 30, 36, 196) found on one end of epsilon toxin (see Figure 15-1) are surface-accessible and important for binding to HAVCR1.[62] Although these data advance our understanding of epsilon toxin–receptor interactions and are potentially useful for therapeutic agent and vaccine development, additional studies are needed to clarify in further detail these cell-surface interactions.

Epsilon toxin is naturally found in the veterinary arena and efficacious vaccines are commonly used in the field, as explained earlier.[63–65] Hyperimmune sera can also afford temporary, passive protection for 3 to 4 weeks in weaned lambs; however, animals showing clinical signs of epsilon intoxication are not typically afforded protection by antitoxin immunoglobulins.[66] It is also possible that a monoclonal antibody targeting a critical epitope, like the membrane insertion region of epsilon toxin, could be a more useful therapeutic, better characterized and purified, than polyclonal antibodies.[67] Immunoglobulins derived by either active or passive immunization are effective tools against *C perfringens* epsilon toxin when present before or after toxin exposure.

Historically, vaccines for humans and animals have proven remarkably effective against myriad diseases throughout the world. However, as with many veterinary vaccines, those for *C perfringens* and associated toxins are often formaldehyde toxoids consisting of various antigens from culture filtrates or whole cells. These vaccines are efficacious and cost effective for animals, but considered too crude for humans.[68] Furthermore, current veterinary vaccines containing epsilon toxoid can vary in immunogenicity and ultimately protective efficacy.[64] The vaccination regimen also varies depending on the animal species.[65] Typically, two doses are given within 2 to 6 weeks of each other using aluminum hydroxide adjuvant, followed by an annual (sheep) or quarterly (goat) boost. In animals, and perhaps in a very heterogeneous population like humans, an epsilon toxin vaccine is clearly not a "one and done" scenario for lasting, protective immunity.

Recombinantly produced epsilon toxin that is subsequently inactivated by 0.5% formaldehyde can be used as a superior cost-effective vaccine (eliciting a sustained, higher antitoxin titer) at much lower protein doses than standard epsilon toxoids.[64] Use of a more defined (purified) vaccine, as opposed to a crude culture filtrate, also affords easier quality control. The gene for epsilon toxin was first successfully cloned, sequenced, and expressed in 1992, making subsequent recombinant work possible.[69] Any human vaccine against epsilon toxin will likely be chemically or recombinantly mutated critical amino acids for receptor binding, oligomerization, or channel formation detoxified versions of the purified protein. In terms of recombinant protein, replacing histidine 106 with proline results in a nontoxic form of epsilon toxin[70] that provides vaccine-based protection against 1,000 LD_{50} of wild-type toxin given intravenously to mice. Further recombinant work could be done using data derived from earlier chemical modifications of epsilon toxin.[71] X-ray crystallography of a toxin-receptor complex would likely yield definitively useful data, leading to an even better recombinant vaccine or novel therapeutics. Medical management of epsilon intoxication, particularly in humans, is currently a wide-open field, not only for physicians but also microbiologists, biochemists, and immunologists.

SUMMARY

C perfringens is a very "toxic" bacterium, employing various proteins to promote life in (and out of) various mammals. Myriad proteins are toxins intimately linked to many *C perfringens* diseases in humans and animals; in particular, the epsilon toxin has been studied by various groups around the world and is primarily a veterinary concern. Vaccines are available for veterinary use, but an equivalent has not been generated for human use in biodefense. From a therapeutic or short-term prophylactic perspective, toxin-specific immunoglobulins should be of logical interest for human use. However, critical experiments employing characterized immunoglobulins (monoclonal and especially those humanized) are lacking in the literature. Simply put, countermeasures for epsilon intoxication in humans are currently highly experimental.

Finally, military and civilian physicians throughout time have been concerned with soiled penetrating wounds involving muscle tissue for fear of gas gangrene due to *C perfringens* (and other anaerobes). More recently, biodefense in the 21st century has targeted a *C perfringens* toxin, epsilon, as a potential nefarious agent. As the discovery of *C perfringens* over 120 years ago suggests, along with subsequent work on the various virulence factors of this extraordinarily toxic bacterium, a collaborative international effort propelled by scientific endeavor is key to discovering knowledge-based medical interventions.

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Chapter 16

RICIN

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SUMMARY

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INTRODUCTION

Ricin is a potent toxin derived from the ornamental and widely cultivated castor plant, *Ricinus communis L* (*Euphorbiaceae*). Ricin is mostly concentrated in the seed of the plant—popularly known as the castor bean—that, despite its name, is not a true bean. The purified ricin toxin is a white powder that is water soluble; it inhibits protein synthesis leading to cell death. Ricin is stable under normal conditions, but can be inactivated by heat exceeding 80°C. After oil extraction and inactivation of ricin, the defatted mash and seed husks are used as animal feed supplement and fertilizer, respectively.[1]

In 1978, the lethality of ricin was overtly established after the high-profile assassination of Bulgarian dissident Georgi Markov.[2] Numerous incidents involving ricin or castor seeds for nefarious purposes have been reported since 1978.[3-9] More recently, various extremists and terrorist groups have also experimented with ricin; some involved mailing ricin-tainted letters to the offices of US politicians. These events have heightened concerns regarding ricin's potential for urban bioterrorism, and thus prompted its constant inclusion in weapons of mass destruction investigations.[10] The wide availability of the castor plants; the relative ease of toxin production; and the toxin's lethality, stability, and media coverage fortify the appeal of ricin for those in quest of retribution and public attention.

In the United States, the possession or transfer of ricin and genes encoding its functional form is regulated by the Centers for Disease Control and Prevention (CDC) Select Agents and Toxins Program. CDC has classified ricin as a category B threat agent. Category B agents, which are the second highest priority agents, are moderately easy to disseminate, result in moderate morbidity and low mortality rates, and require specific enhancements of CDC's diagnostic capacity and enhanced disease surveillance.[11,12] Investigators must register with the CDC before using nonexempt quantities of ricin in their research. No federal regulations restrict the possession of castor plants; however, some states or cities (eg, Hayward, CA) prohibit possession of castor plants or seeds.

Ricin is listed as a schedule 1 toxic chemical under both the 1972 Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on their Destruction, usually referred to as the Biological Weapons Convention, or Biological and Toxin Weapons Convention, and the 1997 Chemical Weapons Convention.[13,14]

HISTORY, BIOLOGICAL WARFARE, AND TERRORISM

History

The castor plant, also known as the Palm of Christ, was initially indigenous to the southeastern Mediterranean region, eastern Africa, and India, but is now widespread throughout temperate and subtropical regions.[15–17] *Ricinus* is a Latin word for "tick" to describe the castor seed's appearance that resembles a tick (*Ixodes ricinus*), and *communis* meaning "common" to describe its worldwide distribution. For centuries, the castor plant has been cultivated for numerous economically important products, primarily castor oil.[17] In ancient Egypt, Europe, India, and China, castor oil was used for lighting and body ointments, and as purgative or cathartic, and other ethnomedical uses. Castor oil was also reportedly used as an instrument of coercion by the Italian Squadristi, the Fascist armed squads of Benito Mussolini.[18] Political dissidents and regime opponents were forced to ingest large amounts of castor oil, triggering severe diarrhea and dehydration that often led to death.[19] Presently, castor oil has abundant commercial applications including medicinal and industrial purposes.[20–24] Castor seeds are being produced in more than 30 countries in the world because of their economic benefits and myriad uses. In 2013, world castor oil seed production totaled 1.86 metric tons, and the leading producers include India, China (mainland), and Mozambique.[25]

In 1888, Peter Hermann Stillmark, a student at the Dorpat University in Estonia (Stillmark 1888, as cited in Franz and Jaax[5]), discovered ricin. During Stillmark's extensive research, he observed that ricin caused agglutination of erythrocytes and precipitation of serum proteins.[17] In 1891, Paul Ehrlich studied ricin and abrin in pioneering research that is now recognized as the foundation of immunology.[17] Ehrlich found that animals vaccinated with small oral doses of castor beans were protected against a lethal dose of the toxin. Additional experiments using abrin and ricin showed that the immunity was specific, was associated with serum proteins, and could be transferred to the offspring through milk. Further research on ricin showed that the toxin described by Stillmark was actually two proteins, one with an agglutinin with a molecular weight of 120 kDa (*R communis agglutinin I*) possessing little toxicity and the other, *R communis agglutinin II*, a smaller molecular weight protein (60 kDa) with little agglutinating capacity

but extremely toxic. Nearly a century after Stillmark's original discovery, Olsnes and Pihl[26] demonstrated that the 60 kDa toxic protein (ricin) inhibited protein synthesis and that the 60S ribosomal subunit is the toxin's molecular target.[27]

Although ricin is considered a possible biological threat (see below), its potential medical applications have been also explored. During the past decade, ricin has been used extensively in the design of therapeutic immunotoxins, often called "magic bullets." Specifically, ricin, ricin A chain (RTA), or a related toxin is chemically or genetically linked to a binding ligand such as an antibody or used in other conjugates to specifically target and destroy cancer cells, and also as alternative therapies for AIDS and other illnesses.[28–30] Ricin-based immunotoxins conjugated to either the anti-CD22 antibody RFB4[31,32] or its Fab fragment[33] have been reported to provide enhanced therapeutic efficacy and improved antitumor activity.[34–36] However, the US Food and Drug Administration has placed a hold on the clinical testing of RTA-based immunotoxins because they caused vascular leak syndrome (VLS) in humans, a life-threatening condition in which fluids leak from blood vessels leading to hypoalbumina, weight gain, and pulmonary edema.[37] Although progress has been made in understanding the mechanisms of immunotoxin-mediated VLS, significant effort is still required to understand VLS and generate RTA-derived immunotoxins that do not cause VLS but also maintain RTA's potent antitumor activity.[38]

Ricin as a Biological Weapon

During World War I, the United States, which was aware of the German biological warfare program, examined ricin for retaliatory intentions.[39] Two methods of ricin dissemination were described in a 1918 technical report: (1) adhering ricin to shrapnel bullets for containment in an artillery shell, and (2) production of a ricin dust cloud (Hunt et al, 1918, as cited in Smart[39]). The thermal instability of ricin constrained its initial use in exploding shells, and ethical and treaty issues limited its use as a poison or blinding agents. World War I ended before the toxin could be weaponized and tested. During World War II, ricin was evidently never used in battle despite its mass production and being armed into ricin-containing bombs (also known as W bombs), because its toxicity was surpassed by the even more potent biological agents of the time.[5] Interest in ricin diminished with the production and weaponization of other chemical agents, for example, sarin. During the Cold War, the Soviet Union studied ricin as a possible biological weapons agent. A former top Russian official who defected to the United States in 1991 asserted that Russia developed ricin as a weapon, and that the toxin used against the Bulgarian dissidents Georgi Markov and Vladimir Kostov was formulated in Russian laboratories.[40] Iraq reportedly manufactured and tested ricin in animals and used it as payload in artillery shells.[41–44] Syria and Iran were believed to have produced unknown quantities of the toxin.[45] Ricin was also found in Afghanistan in 2001 after the collapse of the Taliban government.[46,47]

Although ricin's potential use as a military weapon was investigated, its utility over conventional weaponry remains ambiguous. Despite its toxicity, ricin is less potent than other agents such as botulinum neurotoxin or anthrax. It has been estimated that eight metric tons of ricin would have to be aerosolized over a 100 km^2 area to achieve about 50% casualty, whereas only kilogram quantities of anthrax spores would cause the same effect.[48] Furthermore, wide-scale dispersal of ricin is logistically impractical. Thus, while ricin is relatively easy to produce, it is not as likely to cause as many casualties as other agents.[49]

Ricin as a Terrorist Weapon and Use in Biocrimes

The well-publicized "umbrella murder" of the Bulgarian writer and journalist Georgi Markov in 1978[2] represents the first documented case of a modern assassination using a biological agent,[50] although this remained unproven. Markov defected to the West in 1969 and was a vocal critic of the Bulgarian communist regime. The Bulgarian secret police had previously attempted to kill Markov twice, but failed. However, on September 7, 1978, Markov was assaulted with an umbrella tip while waiting at a bus stop in London. He subsequently developed severe gastroenteritis and a high fever, and died on September 11, 1978. The autopsy revealed a small platinum pellet with an X-shaped cavity. Further examination of the pellet revealed ricin.[2,51] Prosecutors have failed to identify, arrest, or charge anyone for the crime. On September 11, 2013, a news report disclosed that Bulgaria was abandoning its investigations of the notorious case 35 years after the cessation of the absolute statute of limitations.[52]

Days before Markov's assassination, an attempt was made to kill another Bulgarian defector, Vladimir Kostov.[2] However, the pellet lodged in the fatty tissue in Kostov's back prevented the toxin from being released from the sugar-coated pellet; he survived the incident. Several cases involving the possession, experimentation, or planned misuse of ricin by bioterrorists and extremist groups have been investigated or prosecuted by law enforcement agencies worldwide.[5-7] Recent related incidents include the following:

- In April 2013, letters containing ricin were mailed to Republican Senator Roger Wicker (of Mississippi) and the White House.[53,54] A Mississippi martial arts instructor, Everett Dutschke, was charged with sending those ricin-tainted letters.[55]
- In May 2013, five letters, three of which that tested positive for ricin, were mailed from Spokane, Washington, to a local judge, downtown Spokane post office, President Barack Obama, Fairchild Air Force Base, and the Central Intelligence Agency in McLean, Virginia.[56]
- On June 7, 2013, actress Shannon Richardson was arrested for allegedly sending ricin-tainted letters to New York Mayor Michael Bloomberg and President Barack Obama.[57]

These reports further substantiate ricin's image as an attractive lethal poison, and ostensibly, a biological weapon of choice by extremist groups and individuals.

DESCRIPTION OF THE AGENT

Biochemistry

While ricin is a well-known toxin that can be extracted from castor bean mash, most do not realize that it is related in structure and function to the bacterial Shiga toxins and Shiga-like toxin (also known as Verotoxin) of *Shigella dysenteriae* and *Escherichia coli*. Antibiotic-resistant Shiga toxin-producing *E coli* was responsible for 54 deaths in Germany in 2011[58]; the Shiga toxin gene encoding the toxin was carried by this infectious pathogen. Ricin is noninfectious; however, both the structure and enzymatic activities of ricin and Shiga toxins are similar (Figure 16-1). These protein toxins belong to a family of toxins known as ribosome inactivating proteins (RIPs). More than 60 different plant and bacterial species produce RIPs.[59–61] Type I and II RIPs include the plant toxins ricin, abrin, mistletoe lectins, volkensin, modeccin, saporin, trichosanthin, luffin, and the bacterial Shiga toxin and Shiga-like toxin.

Ricin, a type II RIP, consists of two glycoprotein subunits: a catalytic A-chain (RTA) and a lectin B-chain (RTB), which binds cell surface oligosaccharides containing galactose.[59,62–64] The RTA and the RTB, which are of approximately equal molecular mass (~32 kDa), are covalently linked by a single disulfide bond. The protein-coding region of ricin consists of a 24 amino acid N-terminal signal sequence preceding a 267 amino acid RTA. The RTB has 262 amino acids. It consists of two major domains with identical folding topologies,[62] each of which comprises three homologous subdomains (α, β, γ) that probably arose by gene duplication from a primordial carbohydrate recognition domain.[65] RTB binds terminal β 1,4, linked galactose and *N*-acetyl galactosamine (Gal/GalNac)[66] that are on the surface of most mammalian cells. A 12-amino acid linker in the pre-protein joins the two chains. The carboxyl-terminal end of the RTA folds into a domain that interacts with the two domains of the B chain.[62]

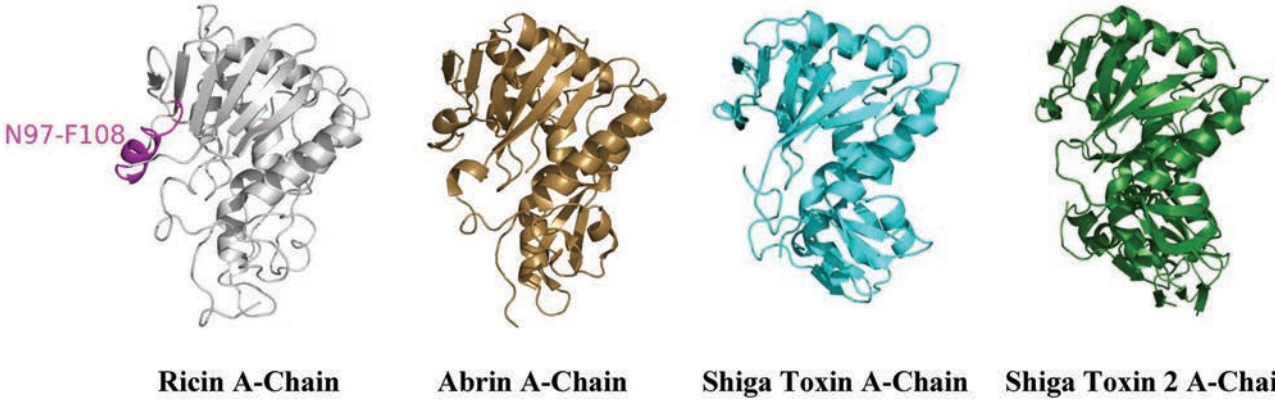


Figure 16-1. Structural and functional similarities among RIPs. The A-chains of plant RIPs such as ricin (PDB 3HIO) and abrin (PDB 1ABR) are structurally and functionally related to the bacterial Shiga toxin A-chains (PDB 1R4Q and 1R4P). The A-chains catalyze the same reaction to inactivate ribosomes and halt protein synthesis.
Data sources: (1) Ho MC, Sturm MB, Almo SC, Schramm VL. Transition state analogues in structures of ricin and saporin ribosome-inactivating proteins. *Proc Natl Acad Sci U S A*. 2009;106:20276–20281. (2) Tahirov TH, Lu TH, Liaw YC, Chen YL, Lin JY. Crystal structure of abrin-a at 2.14 A. *J Mol Biol*. 1995;250:354–367. (3) Fraser ME, Fujinaga M, Cherney MM, et al. Structure of shiga toxin type 2 (Stx2) from *Escherichia coli* O157:H7. *J Biol Chem*. 2004;279:27511–27517.

A disulfide bond is formed between amino acid 259 of the RTA and amino acid 4 of the RTB.[59,63,64] Thirty percent of the RTA protein is helical. The RTA folds into three somewhat arbitrary domains. The active site cleft of the RTA is located at the interface between all three domains.

Pathogenesis

Pathogenesis resulting from ricin intoxication is a two-step process. The first phase occurs at the cellular level in which the toxin kills cells in a cell-cycle independent fashion; the second phase is primarily caused by systemic reactions that develop in response to cell death and tissue damage.

The cell binding component of the toxin (RTB) binds to cell surface lipids and proteins with exposed terminal β-1,4-linked galactose molecules that are found on most mammalian cells, permitting ricin to bind indiscriminately to most cells in the body.[17,66,67] In addition to binding to surface glycoproteins, ricin contains three mannose oligosaccharide chains, two in RTB and one in RTA, that provide another route for ricin binding to the cell via mannose receptors located primarily on macrophages and dendritic cells.[68]

Once RTB binds to the cell, it is endocytosed.[69] At this point, three possible fates exist for ricin:

1. entry into endosomes and recycling to the cell surface;
2. degradation via the late endosomes; or
3. entry to the trans-Golgi network and entry to the endoplasmic reticulum (ER) via retrograde trafficking.

In the ER, a protein disulfide isomerase reduces the toxin into RTA and RTB components.[70] The low lysine content of RTA probably enables the molecule to evade the ER-associated protein degradation pathway and chaperone proteins, such as calreticulin, and to transport RTA from the Golgi apparatus to the ER; dislocation of RTA from the ER may involve the translocon component Sec61p.[71–73] Postdislocation of RTA in the cytosol probably involves Hsp70, which may also aid the protein in binding to its ribosomal substrate.[74] Additionally, the ribosome itself may act as a suicidal chaperone by facilitating proper refolding of RTA, which is required for the catalytic activity of the enzyme.[73]

Extensive investigations on reactions controlling RTA's binding to ribosomes provide detailed information on RTA's enzymatic functions.[17,75] RTA catalyzes the hydrolysis of a specific adenine in the ricin-sarcin loop of the 28S ribosomal RNA (rRNA) (Figure 16-2). The ricin-sarcin loop interacts with eukaryotic elongation factor EF-2. The binding of EF-2 to the ricin-sarcin loop is required for the translocation of the peptidyl-tRNA from the A-site to the P-site on the ribosome during protein synthesis. The depurinated ricin-sarcin loop fails to bind EF-2 and the ribosome stalls with the peptidyl-tRNA stuck in the A-site.[76,77] The overall effect is a halt in protein translation and cell death.

Analysis of reactions resulting from mixing purified rat ribosomes with RTA shows that the RTA reaction follows classical Michaelis-Menten enzyme kinetics, and the enzymatic action has been calculated to be 0.1 mol/L.[78] Furthermore, these studies predict that one RTA molecule would depurinate 1,500 ribosomes per minute, thus making one ricin molecule sufficient to kill the cell. Site-directed mutagenesis and the development of transition state mimics have yielded mechanistic information. The hydrolysis reaction catalyzed by RTA is thought to proceed via a dissociative mechanism with an oxocarbenium transition state.[79] Glu-177 in the active site stabilizes the developing positive charge on the ribosyl ring while Tyr-80 and Tyr-123 have been proposed to activate the leaving group by pi-stacking with the adenine[80] (Figure 16-3). The enzymatic activity of RTA is the primary source of toxicity and therefore must be attenuated in RTA subunit vaccines by incorporating the Y80A mutation[81] or removing the C-terminal residues (residues 199–267).[82] The mutations interfere with rRNA binding.

Activation of apoptotic processes is one method by which RTA kills cells, but the apoptotic pathways are somewhat cell dependent.[81–83] Evidence indicates that some cells have novel ricin-specific pathways for activating apoptosis. Wu and colleagues[84] found that RTA binds to a novel binding protein (BAT3) that is found in the cytoplasm and nucleus of many cells. BAT3 possesses a canonical caspase-3 cleavage site that appears to be exposed when RTA binds to BAT3; apoptosis is then activated with caspase-3 cleavage. The finding that BAT3 may play a role in ricin-induced apoptosis could identify new targets for preventing ricin toxicity.

Ricin intoxication has been shown to activate numerous signaling pathways including mitogen-activated protein (MAP) kinases and subsequent secondary signaling pathways, such as the stress activated protein kinase family.[85] MAP kinases regulate activation of cytokines such as interleukin (IL)-8, IL-1β, and tumor necrosis factor-α that, in turn, cause inflammatory reactions and tissue damage. Although inflammatory responses caused by ricin have been described previously, pathways and resulting cellular responses were only recently examined.[86] Korcheva et al[87] demonstrated that intravenous administration

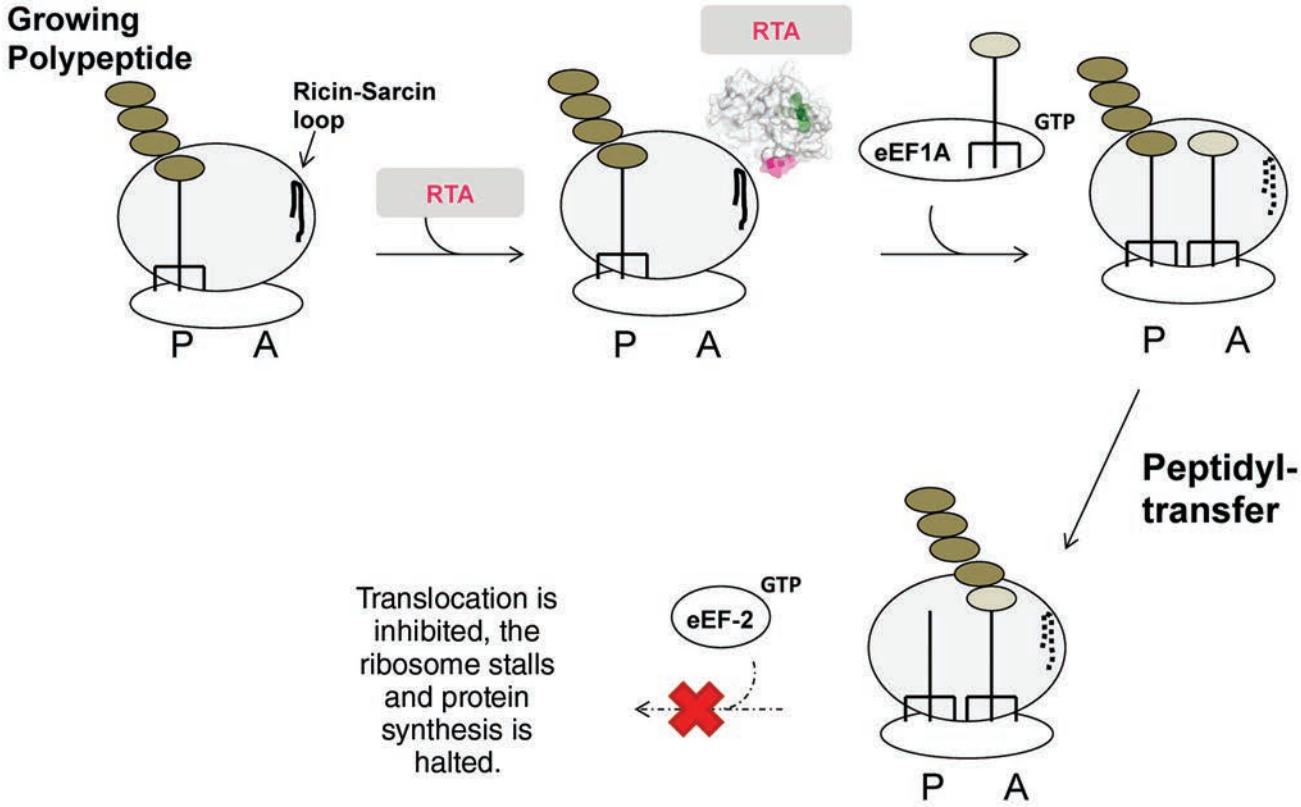


Figure 16-2. The ricin A chain catalyzes the hydrolysis of an adenine in the ricin-sarcin loop. The depurinated rRNA is shown as a dotted line. The aminoacyl-tRNA is delivered to the A-site by eukaryotic elongation factor eEF1A and peptidyl transfer follows. The binding of eukaryotic elongation factor eEF-2 carrying GTP is required for the peptidyl-tRNA to translocation from the A-site to the P-site; this movement requires eEF-2. The depurinated loop fails to bind eEF-2, and the ribosome stalls with the peptidyl-tRNA in the A-site. GTP: guanosine triphosphate
Data source: Figure adapted from Mansouri S, Nourollahzadeh E, Hudak KA. Pokeweed antiviral protein depurinates the sarcin/ricin loop of the rRNA prior to binding of aminoacyl-tRNA to the ribosomal A-site. *RNA*. 2006;12:1683–1692.

of ricin in mice resulted in cellular signaling pathway activation and a significant increase in serum proinflammatory cytokine levels. Additional research in which ricin was instilled by an intratracheal route showed similar signaling pathway activation as well as an increase in proinflammatory cytokine levels, although more inflammatory reactions and tissue damage were observed in the lungs.[88] Although these studies have initiated the systemic pathogenesis characterization of ricin intoxication, further efforts aimed to determine the cellular responses induced by ricin will lead to a greater understanding of its pathogenesis and may also enable the development of new treatment strategies to combat the effects of intoxication.

CLINICAL SYMPTOMS, SIGNS, AND PATHOLOGY

Experimental animal studies reveal that clinical signs and pathological manifestations of ricin toxicity depend on the dose as well as the route of exposure.[5,27,89] The common routes of entry are oral intoxication (ingestion), injection, and inhalation. The differences observed in pathology among various routes likely result from the fact that RTB binds to a wide array of cell surface carbohydrates.[90] Once bound, RTA is internalized and results in the death of intoxicated cells. Although symptoms may vary, in most cases, there is a time-to-death delay of approximately 10 hours, even with a high dose of toxin.[91] Additionally, in animals and humans intoxicated either by injection or oral ingestion, a transient leukocytosis is commonly observed, with leukocyte counts rising two to five times above their normal values. The LD_{50} and time to death for animals by various routes have been reported, and the values for humans were estimated based on animal experiments and accidental human exposures.[5,7,40]

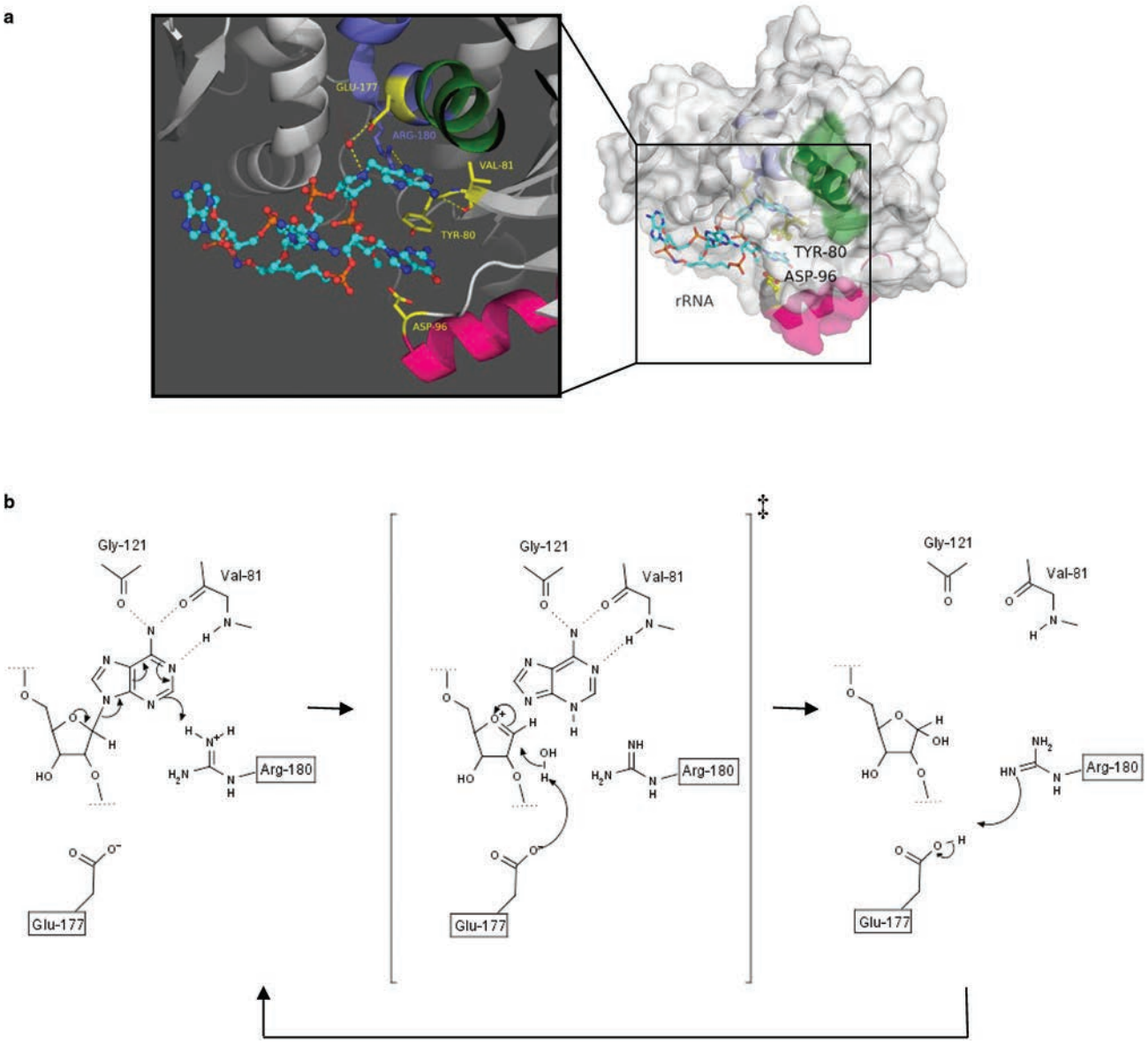


Figure 16-3. RTA catalyzed depurination reaction. (**a**) Structure of a cyclic G(9-DA)GA 2′-OMe transition state mimic determined by Ho et al. (PDB 3HIO). A methylene carbon between the nitrogen of the aza-sugar and the adenine mimics the increased riobsyl-adenine distance in the dissociative transition state. (**b**) Proposed mechanism of the RTA catalyzed depurination reaction. The hydrolysis reaction is thought to proceed via a dissociative mechanism with an oxocarbenium transition state. Arg-180 protonates the leaving group (adenine) and the N-glycosidic bond is broken. Glu-177 deprotonates the hydrolytic water that attacks at carbon to complete the depurination reaction.
Data sources: (1) Ho MC, Sturm MB, Almo SC, Schramm VL. Transition state analogues in structures of ricin and saporin ribosome-inactivating proteins. *Proc Natl Acad Sci U S A*. 2009;106:20276–20281. (2) Roday S, Amukele T, Evans GB, Tyler PC, Furneaux RH, Schramm VL. Inhibition of ricin A-chain with pyrrolidine mimics of the oxacarbenium ion transition state. *Biochemistry*. 2004;43:4923–4933.

Oral Intoxication

Oral or intragastric delivery is the least effective and least toxic route, reportedly 1,000 times less toxic than parenteral routes. The reduction in toxicity may result from poor absorption of the toxin across the epithelium and slight enzymatic degradation of the toxin as it traverses the gastrointestinal tract. Ingestion of castor beans is the most common route of poisoning for humans and domestic animals. Worbs and

colleagues[92] provide an updated list of ricin intoxication in humans worldwide. Since the late 1880s, 875 cases of accidental poisoning and 13 fatalities were reported in the literature (1.5% death rate); there were 11 intentional poisonings, five of which were fatal (45.5% death rate). In recent years, ricin intoxication resulting from intentional poisoning using mashed seeds or crude preparations of ricin has become a major bioterror weapon as well as a method of suicide.[8,9]

Great variability exists in the effects from seed ingestion, which is probably related to the number of seeds, the degree of mastication that releases ricin from the seeds, the age of the individual, and—to a lesser extent—the cultivar of the castor bean plant.[92,93] In addition, an accurate description of ricin intoxication in humans is complicated by other factors including the presence of other somewhat toxic components, such as the ricin agglutinin protein and the alkaloid ricinine, that is found in castor seeds and crude preparations of ricin.[92] These substances can also cause tissue damage and contribute to pathological manifestations.

Despite numerous differences that may play a role in oral toxicity, all fatal or serious cases appear to have a similar clinical history; a recent case study is presented in this reference (Exhibit 16-1).[94] Within a few hours, an onset of nausea, vomiting, and abdominal pain occurs, which is followed by diarrhea,

EXHIBIT 16-1

CASE REPORT: RICIN POISONING CAUSING DEATH AFTER INGESTION OF HERBAL MEDICINE

A 42-year-old male Saudi patient presented to the emergency department with a 12-hour history of epigastric pain, nausea, repeated attacks of vomiting, chest tightness, and mild nonproductive cough.

These symptoms were preceded by a 5-day history of constipation for which the patient ingested a large amount of a mixture of herbal medicine preparation 2 days before his admission. A review of systems was unremarkable. He had no history of any medical illnesses and medication use except for the herbal medicine. Initial examination showed a mild elevation of temperature (38°C), with generalized abdominal tenderness and hyperactive bowel sounds. His respiratory system examination showed equal bilateral air entry and no added sounds. The rest of his systemic examinations were unremarkable.

Laboratory investigations on admission showed mild leukocytosis of 14 x 10^9/L, a normal platelet count of 200 x 10^9/L, and normal hemoglobin level of 15.8 g/dL. Liver enzymes initially showed mild to moderate elevation of alanine transaminase (ALT) 86 U/L (normal range up to 37 U/L), aspartate transaminase (AST) 252 U/L (normal range up to 40 U/L), and serum lactate dehydrogenase 281 U/L (normal range 72–182 U/L), and the renal function was normal. The initial coagulation profile was impaired as documented by a prolonged prothrombin time (19 seconds, control 12 seconds) and a prolonged activated partial thromboplastin time (56 seconds, control 32 seconds). Electrocardiogram showed a right bundle branch block, and a chest radiograph was normal.

After 4 hours of admission, the abdominal pain became worse, and the patient started showing subcutaneous bleeding at the intravenous sites and upper gastrointestinal bleeding, manifested as hematamesis. The patient was managed by intravenous fluid therapy, fresh frozen plasma and platelet transfusion, and gastric decontamination with activated charcoal. A gastrointestinal consultation was requested in which endoscopy was planned after stabilization of the patient, but was not performed because of the patient's rapid deterioration.

In the second day after admission, his liver enzymes increased to a level of 5980 U/L for ALT and 7010 U/L for AST. Serum albumin was 31 g/L (normal range 38–50 g/L), total protein was 59 g/L (normal range 66–87 g/L), and the platelet count dropped to 85 x 10^9/L. His renal function also deteriorated, elevating the creatinine level to 150 Umol/L (normal range up to 123 Umol/L), and urea to 110 mmol/L (normal range 1.7–83 mmol/L). His blood and sputum cultures and sensitivity were negative for bacterial pathogens, and an abdominal computerized tomography scan was normal. The patient was managed conservatively with supportive measures as maintained earlier; however, he remained persistently hypotensive necessitating inotropic support. On the third day, he developed cardiopulmonary arrest and was resuscitated; however, he could not be revived. The sample of the herbal medicine powder was sent to the university lab. The chemical contents were extracted by the liquid chromatography/mass spectrometry technique, revealing the presence mainly of ricin powder that was further identified by the immuno-polymerase chain reaction assay that confirmed the presence mainly of ricin with no other significant contaminants. This finding could be implicated as the cause for the patient's fulminant clinical course.

Data source: Assiri AS. Ricin poisoning causing death after ingestion of herbal medicine. *Ann Saudi Med*. 2012;32:315–317.

hemorrhage from the anus, anuria, cramps, and pupil dilation.[95] Fever develops, followed by thirst, sore throat, and headache, leading to vascular collapse and shock. Death usually occurs by day three or thereafter. Common histopathological findings during autopsy are multifocal ulcerations and hemorrhages in the stomach and small intestinal mucosa. Significant lymphoid necrosis occurred in intestinal associated lymph nodes, lymphoid tissue, and spleen. Necrosis was also observed in cells of the reticuloendothelial system resulting in liver damage and nephritis. Macrophages and macrophage-derived cells appear to be very susceptible, probably because of the large numbers of mannose receptors present in the cell membrane.[68,96]

Injection

Pathological damage caused by injection of ricin depends largely upon the dose. Results of a clinical trial in which 18 to 20 μg/m^2 of ricin were given intravenously to cancer patients indicated that the low dose was fairly well tolerated, with flu-like symptoms, fatigue, and muscular pain as the main side effects.[97] Some patients experienced nausea and vomiting, but after 2 days, they had recovered and experienced no more side effects. At low doses, intramuscular or subcutaneous injections may result in necrosis at the injection site possibly resulting in secondary infections.[98] High doses by either route cause severe local lymphoid necrosis, gastrointestinal hemorrhage, diffuse nephritis, and splenitis. Targosz and colleagues[99] describe a suicide case in which an individual injected himself subcutaneously with a large dose of ricin extracted from castor beans. The 20-year-old man was admitted to the hospital 36 hours after injection. He experienced severe weakness, nausea, dizziness, headache, and chest pain. Clinical exams showed hypotension, anuria, metabolic acidosis, and hematochezia. The patient was observed with hemorrhagic diathesis and liver, kidney, cardiovascular, and respiratory systems failure requiring endotracheal intubation and artificial ventilation. Although given maximal doses of pressor amines and treated for hemorrhagic diathesis, treatments were ineffective and the patient developed symptoms of multiorgan failure followed by asystolic cardiac arrest. Resuscitation was not effective, and the patient died shortly thereafter. A postmortem examination revealed hemorrhagic foci in the brain, myocardium, and pleura.

In the case of Georgi Markov,[2] the lethal injected dose was estimated to be 500 μg. Markov experienced severe local pain after the injection, which was followed by a general weakness 5 hours later. Fifteen to 24 hours later, he had an elevated temperature, felt nauseated, and vomited. He was admitted to the hospital with a high fever and signs of tachycardia. While his blood pressure remained normal, lymph nodes in the affected groin were swollen and sore, and a 6-cm diameter area of induration was observed at the injection site. Just over 2 days after the attack, he suddenly became hypotensive and tachycardic with a pulse rate of 160 beats/minute and a white blood cell count of 26,300/mm.[3] He became anuric developing gastrointestinal hemorrhaging and complete atrioventricular conduction block. Shortly thereafter, Markov died from cardiac failure complicated by pulmonary edema; the time of death was 3 days after he was initially poisoned.[2]

Inhalation

No reports exist in which humans have been subjected to ricin by accidental inhalation or premeditated aerosolized exposure. Most of the human data comes from descriptions of workers being exposed to castor bean dust in or around castor bean processing plants.[100] Allergic manifestations induced by ricin dust were first described in 1914.[101] Symptoms and clinical signs of intoxication were later differentiated from the allergic syndrome and further investigations showed that the allergens and toxin were two different molecules.[102–104]

Because no data exist for human exposure, it is important to determine whether a consistency exists between rodents and nonhuman primates (and other animal models) that can be used to extrapolate an accurate representation of inhalational ricin in humans. Unlike other routes of intoxication, damage caused by an aerosol exposure is greatly dependent on particle size, and to a lesser extent on the dose and cultivar from which ricin was obtained.[86] Ricin extracted from *R communis var. zanzibariensis* was twice as lethal as ricin extracted from the Hale Queen variety.[86] The differences are more than likely related to variations in the isotoxins of ricin found in the seeds from different cultivars.[86]

For ricin to reach the lung, the particles would need to be a size that could move around the nasal turbinates and flow with the airstream to the lung. Roy and colleagues[105] compared the outcome of mice receiving 1 μm versus 5 μm particle size by an aerosol challenge. With the 1 μm particles, the majority of ricin was found in the lung and by 48 hours, lung tissue show significant lesions with alveolar edema, fibrin, and hemorrhage. Seventy-two hours postexposure, all of the mice had died. Conversely, no deaths were observed when mice were exposed to ricin with a 5 μm mass median diameter. Most of the toxin was found in the trachea, and little lung damage was observed in histological sections of lung tissue taken 48 hours postexposure.

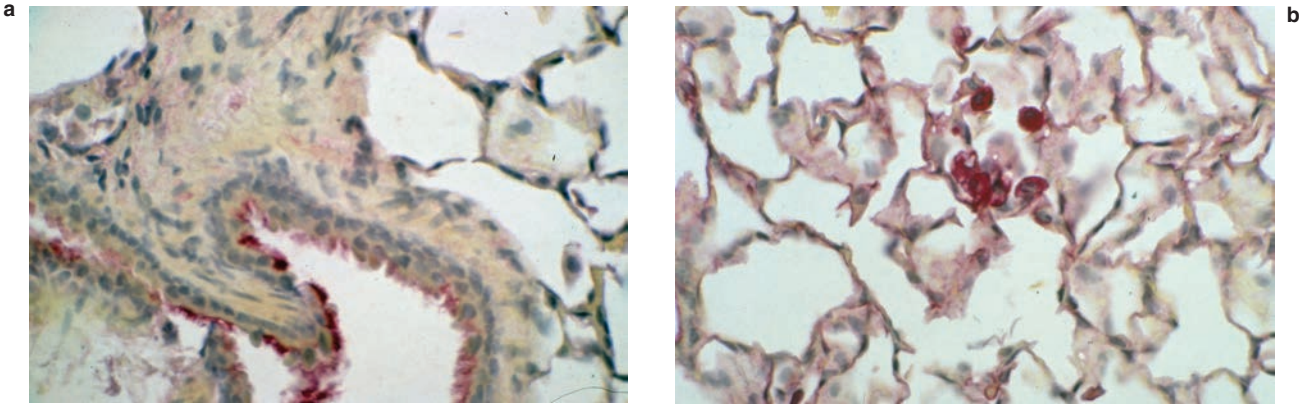


Figure 16-4. Histological sections of lungs from CD1 mice exposed to ricin by aerosol showing (**a**) perivascular edema and pulmonary epithelial necrosis. Hematoxylin and eosin stain at original magnification × 25; (**b**) pulmonary epithelial cell necrosis, hematoxylin and eosin stain at original magnification × 100.
Photographs: Courtesy of Lieutenant Colonel (Retired) Catherine L. Wilhelmsen, Pathology Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

When rats were exposed to a sublethal dose (LCt_{30}) of ricin with particle sizes less than 1 μm, damage was limited to the lung and no histological changes were noted before 8 hours postchallenge.[86,106] By 48 hours, pathological changes observed included necrosis and apoptosis in bronchial epithelium and macrophages present in the alveolae septae. Photographs of tissue sections from CD1 mouse lungs 48 hours after exposure show perivascular edema and pulmonary epithelial cell necrosis (Figure 16-4). Three days post-exposure, there was significant diffuse alveolar edema, and severe capillary congestion and macrophage infiltration of the alveolar interstitium. By day four, there was a rapidly resolving pulmonary edema and renewal of the bronchial epithelium, even though severe pas-sive venous congestion existed in all solid peripheral organs. Fourteen days postexposure, all animals survived. Examination of tissue sections from sacrificed animals were similar to control tissues, except for focal areas of intraalveolar macrophage infiltration.[106] Additionally, when rats and mice were given lethal doses of ricin by aerosol, no indication of lung damage was observed during the first 4 to 6 hours.[14,106,107] By 12 hours, there was an increase in total protein and polymorphonuclear cells in the bronchial lavage, indicating damage to the epithelial cell barrier. Thirty hours after challenge, alveolar flooding was apparent, along with arterial hypoxemia and acidosis. Histopathology showed lesions throughout the respiratory tract, spleen, and thymus. A median lethal dose of ricin by inhalation was determined to be 1 μg/kg body weight for both Sprague Dawley rats and BALB/c mice.[107] Further characterization of inhaled ricin exposure was performed by examining lung tissue sections for the presence of ricin.[5] Immunohistochemical studies showed that ricin binds to the ciliated bronchiolar lin-ing, alveolar macrophages, and alveolar lining cells.[14] This finding further substantiates the importance of the lung epithelium and alveolar macrophages in the inhaled ricin intoxication process.

As with other laboratory animal models, investigations in which nonhuman primates were challenged with an aerosolized dose of ricin indicate that disease progression is proportional to particle size.[108] Inhalational challenge with a particle size of 8 μm was not lethal and did not cause lung damage, suggesting that the upper airways can effectively remove the toxin before it reaches the lung. Inhalational challenge with a particle size of 1 μm presented an entirely different picture with histopathologic changes beginning as early as 4 to 6 hours postexposure.[108,109] By 8 hours, pulmonary changes included alveolar edema, perivascular interstitial edema, lymphangiectasis, alveolar septal necrosis, and hemorrhage. At 16 hours, progression of pulmonary tissue damage continued, and by 24 hours, there was edema, pulmonary congestion, necrotic alveolar septa, and necrotic bronchiolar epithelium (Figure 16-5). Thirty-two hours later, there was marked perivascular and peribronchiolar interstitial edema and alveoli contained fluid (edema) mixed with fibrin and viable or degenerate neutrophils and macrophages. The bronchiolar epithelium was necrotic and often sloughed into the lumen, whereas lymphatics surrounding the airways were moderately dilated and the endothelium of many small vessels had atrophied. In the tracheal mucosa, there was epithelial degeneration with scattered areas of necrosis and subacute inflammation. The cortex of adrenal glands showed mild degeneration and necrosis, and there

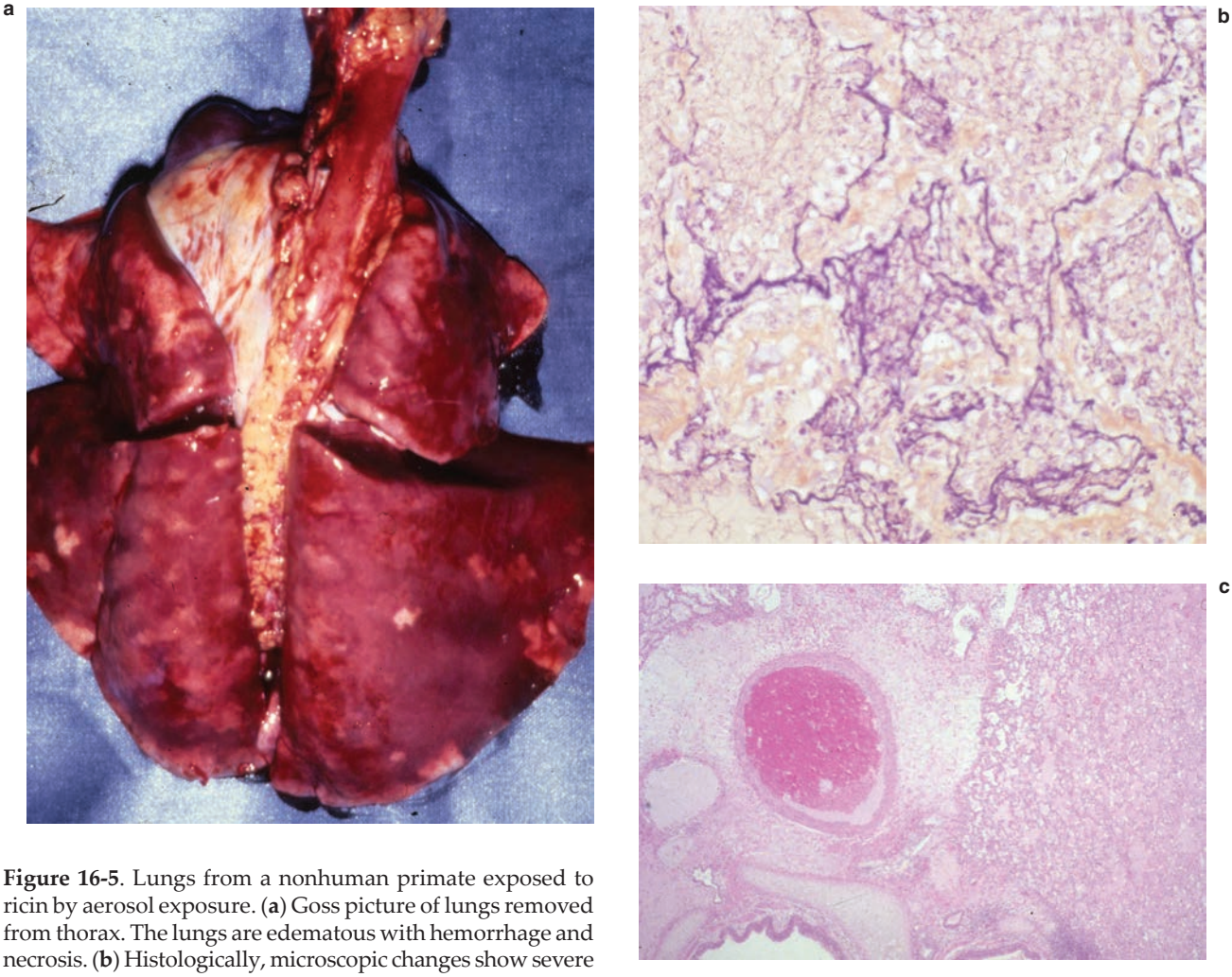


Figure 16-5. Lungs from a nonhuman primate exposed to ricin by aerosol exposure. (**a**) Goss picture of lungs removed from thorax. The lungs are edematous with hemorrhage and necrosis. (**b**) Histologically, microscopic changes show severe perivascular edema; hematoxylin and eosin stain at original magnification × 10. (**c**) Alveolar fibrinopurulent exudate is observed hematoxylin and eosin stain at original magnification × 100. Photographs courtesy of Lieutenant Colonel (Retired) Catherine L Wilhelmsen, DVM, PhD, Pathology Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

was lymphoid depletion and lymphocytolysis in the mediastinal lymph nodes. A similar course of disease was observed in an earlier study in which nonhuman primates were challenged with ricin (~1 μm particle size), but the preclinical period varied between 8 and 24 hours in relation to the size of the original challenge dose.[109] This stage was followed by anorexia and decrease in physical activity. The time of death was also dose dependent and occurred between 36 and 48 hours.

Cause of Death

Although the exact cause of death from ricin toxicity is not known, clinical symptoms of individuals exposed to lethal doses of the toxin suggest that death results from a severe inflammatory response and multiorgan failure.[94,95,99] A lethal dose of ricin given to mice by intraveneous injection or intratracheal instillation results in a systemic inflammatory response, thrombocytopenia, hemolytic anemia, renal failure, and microvascular thrombosis, pathologies that are similar to those observed in humans.[87,88] Initially, the fact that macrophages are extremely sensitive to ricin led investigators to believe that macrophages might play a significant role in ricin intoxication.[110] Recent findings demonstrated that pulmonary inflammation caused by ricin required the presence of both macrophages and interleukin-1 signaling pathways.[111] Also, studies using bone marrow derived macrophages showed that ricin inhibition of protein translation led to activation of IL-1β-dependent inflammation by activating innate immune signaling through the

nod-like receptor (NLR) family member, NLRP3.[112] NLRP3 is an innate immune pattern recognition receptor found in the cytosol that is activated by molecular patterns found on many pathogens or other danger-associated proteins. Activation of NLRP3 stimulates IL-1β processing via a multiprotein complex, the inflammasome. More investigations are necessary to understand how ricin activates severe inflammatory responses that lead to multiorgan failure, shock, and death.

DETECTION AND DIAGNOSIS

Early clinical symptoms of ricin intoxication may resemble symptoms caused by other biothreat agents, and therefore it is essential to identify the etiological agent to provide the best treatment for exposed patients. The cellular uptake of ricin is rapid and thus limits the diagnosis of ricin in blood and other fluid samples. Additionally, the ricin concentration may be below the current levels of detection, making diagnosis more difficult.[113] Because of the inability to detect ricin in patients, identifying the toxin in environmental or forensic samples associated with the exposure remains the most reliable method for determining the presence of ricin and the possibility of intoxication. Ricin does not replicate, so detection relies on the ability to identify physical attributes of the toxin within the sample. The most common method for toxin identification uses antiricin antibodies to which ricin would bind. In recent years, several variations of antigen (toxin)-antibody assays have been developed.[114] Physical characterization using liquid chromatography and mass spectroscopy complements the antibody-based methods and permits development of signatures of the toxin preparation.[115]

Enzyme-linked Immunosorbent Assay

An enzyme-linked immunosorbent assay (ELISA) provides an economical and straightforward method for detecting the presence of ricin in environmental and forensic samples. A capture antibody ELISA is a common method of detection. Ricin is initially "captured" onto the matrix via an antiricin monoclonal antibody (Mab) recognizing RTB. A second anti-ricin Mab, usually recognizing RTA, binds to the immobilized ricin, and the second Mab is then detected by an anti-mouse immunoglobulin 3 conjugated to an enzyme such as horse radish peroxidase that forms a colometric reaction upon the addition of its substrate solution.[86] The limit of detection (LOD) for these assays has been greatly improved by using methods that amplify the detection signal or use a more sensitive signal such as those generated by electrochemiluminescence (ECL). Using slight modifications of these assays, Poli and colleagues[116] reported LODs of 100 pg/100 μL in human serum and urine that had been spiked with various concentrations of ricin. Other studies, such as those by Roy et al,[105] detected ricin in lungs, stomach, trachea, and nares using an ELISA based on time-resolved fluorescence. Although these colorimetric and ECL methods permit detection of high pg or low ng concentrations, sensitivity issues still exist, particularly when assessing foods or biological tissues. Recently, an immuno-polymerase chain reaction assay that uses a polymerase chain reaction to amplify a DNA-labeled reporter system bound to the anti-mouse immunoglobulin G (IgG) permits accurate detection of ricin in these biologic samples ranging from 1 pg/mL to 100 pg/mL.[117] The immuno-polymerase chain reaction may not only offer a method that greatly enhances the ability to detect ricin in environmental samples, but also and more importantly it provides a technique that will accurately determine ricin in tissues from individuals exposed to ricin.

Handheld Assay Detection Devices

Although routine capture ELISAs provide accurate diagnostic tools, these assays require a laboratory setting and instruments to measure the signal. Antibody-based handheld assay (HHA) devices were developed to enable first responders to assess the situation in the field.[118] HHAs were initially developed to detect the anthrax in the letters sent through the mail to Senator Tom Daschle's office in 2001. The success of anthrax spore identification initiated development of HHAs for ricin and other biothreat agents. In 2004, HHAs identified ricin in letters sent to the Dirksen Senate Office building.[118] For ricin, HHAs have an antiricin Mab bound as a single line on the matrix bed. The sample is added to one end of the bed, and capillary action causes the sample to flow across the matrix. The toxin binds to the antibody and then another detection antibody is added. If ricin is present, then the detecting antibody causes color development at the line. If samples are positive using a HHA, samples are sent to a laboratory for confirmation and further analysis.

Sample Verification Platforms

Laboratories, such as the US Army Medical Research Institute of Infectious Diseases (USAMRIID) and the National Biodefense Analysis and Countermeasure Center, need capabilities that will accurately

identify ricin. Multiple instrumental platforms with ELISA-based formats have been developed, including the following:

- ECL-based ricin immunoassay (LOD, 0.05 ng/mL);
- Luminex MAGPIX multiplex (LOD, 0.001 ng/mL); and
- MesoScale Discovery (MSD) PR2 Model 1900 ECL (LOD, 0.2 ng/mL).

Both the M1M ECL-based ricin immunoassay and the Luminex MAGPIX use magnetic beads that are labeled with antiricin antibodies.[119] Once ricin binds to the magnetic beads via the antibody, the sample is sent through the instrument where the magnetic beads are captured by an internal magnet. The magnet is set on an electrode that delivers the proper amount of electrical potential resulting in the emission of light identifying that the sample contains ricin. The MSD PR2, which is a highly sensitive ELISA, has the advantage of using less sample amount (25 μL). The detection antibody is conjugated to a chemiluminescent label that allows for ricin detection by ECL.

On May 30, 2013, a multiplexed rapid ricin detection assay was launched by Radix BioSolutions Ltd (Austin, TX) through the CDC's Laboratory Response Network.[120] This assay was developed using Luminex xMAP technology that permits concurrent detection of several agents in an adaptable, multiplexed assay architecture. Following this report, Tetracore Inc (Rockville, MD) publicized the successful study completion and validation of its BioThreat Alert Lateral Flow Assay and BioThreat Alert Lateral Flow Assay Reader by the Department of Homeland Security for ricin detection.[121]

Liquid Chromatography/Mass Spectrometry

Another method for ricin detection includes identification by liquid chromatography/mass spectrometry. The combination of liquid chromatography and mass spectrometry allows for the separation of mixtures in a sample while being able to identify specific substances based on their molecular mass via their mass to charge ratio (m/z).[122] The ionization of the molecules in the sample can either be protonated or deprotonated depending on the characteristics of the analyte and the mode of detection.[123,124] The advantage of using this technique allows for ricin detection when very little sample is available. Picogram amounts of ricin can be detected within a 5-hour timeframe allowing for fast, reliable detection.[125] Liquid chromatography/mass spectrometry can also be used to characterize other components within the sample because they may provide "signatures" that suggest the origin of the agent. For example, a highly pure form of the toxin might indicate that an organized terrorist group, such as Al Qaeda, produced the ricin while a less pure form may indicate fewer organized groups or individuals acting alone.[86]

Ricin Activity Assay

When ricin is detected using an ELISA or other physical types of assays, the ability to determine whether the toxin is active becomes important for forensic evidence. The assay itself needs to accurately detect ricin's biological activity in samples of limited size (about 50 mL) and low toxin concentration (about 10 ng/mL), and preferably, with an assay time less than 6 hours. To meet these criteria, a cell-free translation (CFT) assay was developed at USAMRIID.[126] The CFT assay measures luminescence generated by the enzyme luciferase produced from the translation of luciferase m-RNA in a rabbit reticulocyte lysate system. The amount of luminescence, produced when the luciferin substrate is added to luciferase, is proportional to the amount of luciferase produced in the in vitro translation system. When ricin is added to the mixture, translation of luciferase mRNA is reduced, which decreases the amount of luciferase produced. Since the amount of luminescence developed is proportional to the amount of luciferase present in the CFT mixture, a reduction in luminescence, as compared to a ricin standard control, provides a quantitative assessment of active ricin in the sample.[126] Table 16-1 summarizes the most commonly used techniques for ricin detection and their sensitivity limits.

Diagnosis

Diagnosis of ricin intoxication is challenging because the cellular uptake of ricin is extremely rapid and limits the availability of ricin for diagnosis in blood and other fluid samples to 24 hours postintoxication.[113] Experimental data suggest that the plasma half-life of ricin is biphasic with the early α phase half-life lasting approximately 4 minutes; the longer β phase half-life was determined to last approximately 83 minutes. The biphasic half-life suggests rapid distribution and uptake of the toxin followed by the slow clearance of excess toxin.[113] Additional liquid chromatography/mass spectrometry assessment of urine samples for metabolites, particularly alkaloids such as ricinine that are commonly found in ricin preparations, indicates ricin intoxication if the individual has symptoms

TABLE 16-1

BIOCHEMICAL METHODS FOR RICIN DETECTION AND THEIR LIMITS OF SENSITIVITY

Method*	LOD† (ng/mL)	Time‡	Detection§	Reference
ELISA-based	0.01–10	5–7 h	Ricin	1–4
Handheld	10–50	90 min	Ricin	5
ECL-based ELISA	0.001–10	4–7 h	Ricin	6–8
Immuno-PCR	0.01–0.1	3–5	Ricin	3, 9
LC/MS	0.1–8	5 h	Ricin/ricinine	10–13
CFT	10–50	4–5 h	Biological activity	14

*Each method may include several different assays using similar principles and formats
†The limit of detection is the lowest amount of ricin detected
‡The time required to perform the assay
§The assays detect either the physical form of ricin or determine the biological activity
CFT: cell-free translation
ELISA: enzyme-linked immunosorbent assay
LC/MS: liquid chromatography/mass spectrometry
LOD: limit of detection
PCR: polymerase chain reaction
Data sources: (1) Griffiths GD. Understanding ricin from a defensive viewpoint. *Toxins (Basel)*. 2011;3:1373–1392. (2) Roy CJ, Hale M, Hartings JM, Pitt L, Duniho S. Impact of inhalation exposure modality and particle size on the respiratory deposition of ricin in BALB/c mice. *Inhal Toxicol*. 2003;15:619–638. (3) Bozza WP, Tolleson WH, Rosado LA, Zhang B. Ricin detection: tracking active toxin. *Biotechnol Adv*. 2015;33:117–123. (4) Poli MA, Rivera VR, Hewetson JF, Merrill GA. Detection of ricin by colorimetric and chemiluminescence ELISA. *Toxicon*. 1994;32:1371–1377. (5) Wade MM, Biggs TD, Insalaco JM, et al. Evaluation of handheld assays for the detection of ricin and staphylococcal enterotoxin B in disinfected waters. *Int J Microbiol*. 2011;2011:132627. (6) DHS fund ricin detection. *Homeland Security News Wire*. October 20, 2011. http://www.homelandsecuritynewswire.com/dhs-funds-ricin-detection. Accessed May 29, 2015. (7) Radix BioSolutions News, 2013. Accessed May 29, 2015. (8) GlobalBiodefense.com. Tetracore Completes DHS Validation of Ricin Detector. http://globalbiodefense.com/2013/06/12/tetracore-completes-validation-of-ricin-detector-for-dhs/. Accessed March 15, 2016. (9) He X, McMahon S, Henderson TD II, Griffey SM, Cheng LW. Ricin toxicokinetics and its sensitive detection in mouse sera or feces using immuno-PCR. *PLoS One*. 2010;55:e12858. (10) Thompson M. High-performance liquid chromatography/mass spectrometry (LC/MS). *AMC Technical Brief*. Analytical Methods Committee AMC TB 34. London, England: Royal Society of Chemistry; 2008. (11) Fredriksson SA, Hulst AG, Artursson E, de Jong AL, Nilsson C, van Baar BL. Forensic identification of neat ricin and of ricin from crude castor bean extracts by mass spectrometry. *Anal Chem*. 2005;15;77:1545–1555. (12) Becher F, Duriez E, Volland H, Tabet JC, Ezan E. Detection of functional ricin by immunoaffinity and liquid chromatography-tandem mass spectrometry. *Anal Chem*. 2007;79:659–665. (13) Kanamori-Kataoka M, Kato H, Uzawa H, et al. Determination of ricin by nano liquid chromatography/mass spectrometry after extraction using lactose-immobilized monolithic silica spin column. *J Mass Spectrom*. 2011;46:821–829. (14) Hale ML. Microtiter-based assay for evaluating the biological activity of ribosome-inactivating proteins. *Pharmacol Toxicol*. 2001;88:255–260.

associated with it.[86] Individuals who survive ricin intoxication develop circulating antibodies in their blood that can be used to confirm intoxication. However, these antibodies are not present until approximately 2 weeks postintoxication and, therefore, could not be used in the initial diagnosis.

MEDICAL MANAGEMENT

Despite the history of ricin's use as a weapon, and unlike other toxin-mediated illnesses such as botulism, no Food and Drug Administration-approved therapeutic for ricin exposure exists. Given that ricin does not have cell specific selectivity, treatment of ricin intoxication is dependent on the site or route of entry, is largely symptomatic, and basically supportive to minimize the poisoning effects of the toxin. Medical countermeasures that have demonstrated capability to disrupt the ricin intoxication process include vaccines and antibody therapy. Both rely on the ability of antibody to prevent the binding of ricin to cell receptors. To ensure maximum protection, the vaccine must be given before exposure, and sufficient antibody must be produced.

Ricin Vaccines

Development of a ricin vaccine has previously focused on either a deglycosylated ricin A chain (dgRTA) or formalin-inactivated toxoid.[127] Although both preparations conferred protection against aerosolized ricin, the proteins aggregated and precipitated over time. Additionally, ricin is not completely inactivated by formalin and may retain some of its enzymatic activity (albeit approximately 1,000-fold lower than native ricin). Thus, other approaches to vaccine development have been investigated to develop a safe and efficacious candidate.

Recent research has focused on developing recombinant RTA subunit vaccines to eliminate cytotoxicity and improve the stability of the vaccine[13] (Figure 16-6). Researchers at the University of Texas developed RiVax that contains the Y80A mutation to inactivate catalysis, and the V76M mutation to ensure the removal of any trace of VLS activity from the immunogen.[128,129] RiVax is at least 10,000-fold less active than wild type RTA but has also been shown to protect rodents against aerosol challenge.[129] In 2006, RiVax was tested in phase I clinical trials. Results of these studies showed that RiVax appeared to be immunogenic and well tolerated in humans.[130,131] However, while such findings were encouraging, vaccine formulation and stability remain problematic. Hence, a lyophilized formulation that retained immunogenicity when stored at 4°C was developed.[132,133] RiVax has been out-licensed to Soligenix (Princeton, NJ) for more advanced clinical trials.[134,135]

To overcome both safety and stability issues simultaneously, researchers at USAMRIID structurally modified the RIP-protein fold of RTA to create a nonfunctional scaffold for presentation of a specific protective epitope.[82] The engineered RTA 1–33/44–198 (RVEc) was produced in *E coli* and lacks the C-terminal residues 199–276 as well as a loop between residues 34–43 (Figure 16-6). RVEc contains a number of well-characterized protective B-cell epitopes, but is more stable and less prone to aggregation. Based on preclinical studies, this product was determined to have a reasonable safety profile for use in human studies; it demonstrated no detectable RIP activity or evidence of VLS.[136–139] In April 2011, USAMRIID launched a phase I escalating, multiple-dose study to evaluate the safety and immunogenicity of RVEc in healthy adults, and it was completed in November 2012.[138] The vaccine was well tolerated and immunogenic.[138,139] In June 2013, a phase 1a (Version 2.0) protocol was implemented as a single-dose, single-center clinical study to allow for the administration and evaluation of a fourth boost vaccination.[139] The ELISA and TNA anti-ricin IgG endpoint titers for the four boosted subjects indicated a robust response very soon after a boost vaccine. In conjunction with this study, another protocol was also started in June 2013 for the collection of plasma from previously RVEc vaccinated subjects for passive transfer studies in animal models to demonstrate IgG as a surrogate marker for clinical efficacy. No adverse events have been reported on this study.[139]

The RVEc final drug product passed stability testing through the 48 months.[139] In addition, the potency assay results confirmed the vaccine elicited protective immunity in mice against 5 times the lethal ricin toxin dose, and it was capable of inducing anti-ricin neutralizing antibodies. An end of clinical use stability testing to include the 54-month time point was initiated in October 2013 for both the final drug product and the diluent.[139]

A comparative immunogenicity and efficacy study between RVEc and RiVax has been conducted in mice.[140] Both candidate RTA vaccines were found equally effective in eliciting protective immunity; however, quantitative differences were observed at the serologic level. RVEc was slightly more effective than RiVax in eliciting ricin-neutralizing antibodies. Furthermore, the antisera elicited by RVEc were toward an immunodominant neutralizing linear epitope on RTA (Y91 to F108), whereas those of RiVax were confined to residues 1–198.[140]

Antibody Treatment

Passive protection with aerosolized antiricin IgG has been evaluated as prophylaxis before aerosol challenge. In mice, pretreatment of nebulized antiricin IgG protected against aerosol exposure to ricin.[141] Preclinical studies also have shown the protection afforded by neutralizing monoclonal antibodies against a lethal dose challenge of ricin.[142–144] Researchers at Defence Science and Technology Laboratory in Porton Down, United Kingdom, have developed polyclonal antiricin antibodies that were raised in sheep immunized with ricin toxoid plus incomplete Freund's adjuvant.[145] The protective efficacy of both IgG and $F(ab')_2$ were demonstrated in mice against ricin intoxication when administered 2 hours following either systemic or inhalational ricin challenge, while the smaller Fab' fragment did not prevent death from ricin intoxication.[145,146] This demonstrates the feasibility of producing an effective ovine antiricin antibody for use following ricin intoxication. In a recent study, four chimeric toxin-neutralizing monoclonal antibodies were produced and evaluated for their ability to passively protect mice from a lethal-dose ricin challenge.[147] The most effective antibody, c-PB10, had the lowest IC_{50} (half-maximal inhibitory concentration) in a cell-based toxin-neutralizing assay and was sufficient to passively protect mice against systemic and aerosol toxin challenge.[147]

The use of antitoxins as therapies for toxin exposure has limitations including the following:

- anaphylactoid or anaphylactic reactions;
- requirement of timely detection of exposure; and
- the therapeutic window is dependent on the toxin and the dose received.[145]

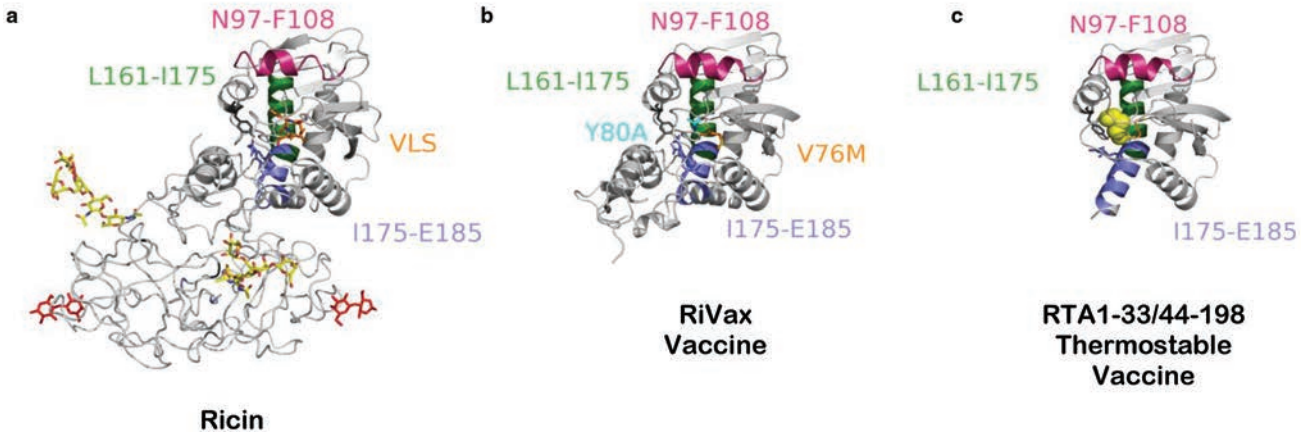


Figure 16-6. Ricin vaccines have been derived from the A-chain of the toxin. (**a**) Ricin consists of an A-chain and a B-chain. The A-chain is shown in *ribbon*, and B-chain in *worm*. (**b**) The ricin A-chain can be produced recombinantly in *Escherichia coli* apart from the B-chain. The structure of RiVax (PDB 3SRP) is similar to the structure of the A-chain of the toxin. (**c**) Truncation of the hydrophobic C-terminal residues of the A-chain and the loop increased the thermal stability of the protein and reduced its propensity to aggregate. The incorporation of disulfide bonds further enhanced the thermal stability of the immunogens (PDB 3MK9, 3LC9, and 4IMV). The protective epitopes are colored on each protein. The UNIVAX R70 epitope is shown in *magenta*; the B-cell epitope recognized by human neutralizing antibodies between Leu161-Ile175 was identified by Castelletti et al (2004) and is shown in *green*. The T-cell epitope between Ile175-Tyr183 is shown in *blue*.
Data sources: (1) Legler PM, Brey RN, Smallshaw JE, Vitetta ES, Millard CB. Structure of RiVax: a recombinant ricin vaccine. *Acta Crystallogr D Biol Crystallogr*. 2011;67(Pt 9):826–830. (2) Compton JR, Legler PM, Clingan BV, Olson MA, Millard CB. Introduction of a disulfide bond leads to stabilization and crystallization of a ricin immunogen. *Proteins*. 2011;7:1048–1060. (3) Janosi L, Compton JR, Legler PM, et al. Disruption of the putative vascular leak peptide sequence in the stabilized ricin vaccine candidate RTA1-33/44-198. *Toxins (Basel)*. 2013;5:224–248. (4) Lemley PV, Amanatides P, Wright DC. Identification and characterization of a monoclonal antibody that neutralizes ricin toxicity *in vitro* and *in vivo*. *Hybridoma*. 1994;13:417–421. (5) Castelletti D, Fracaso G, Righetti S, et al. A dominant linear B-cell epitope of ricin A-chain is the target of a neutralizing antibody response in Hodgkin's lymphoma patients treated with an anti-CD25 immunotoxin. *Clin Exp Immunol*. 2004;136:365–372. (6) Tommasi M, Castelletti D, Pasti M, et al. Identification of ricin A-chain HLA class II-restricted epitopes by human T-cell clones. *Clin Exp Immunol*. 2001;125:391–400.

Supportive and Specific Therapy

The route of exposure for any agent is an important consideration in determining prophylaxis and therapy. For oral intoxication, supportive therapy includes intravenous fluid and electrolyte replacement and monitoring of liver and renal functions. Standard intoxication principles should be followed. Because of the necrotizing action of ricin, gastric lavage or induced emesis should be used cautiously. An aerosol-exposed patient may require the use of positive-pressure ventilator therapy, fluid and electrolyte replacement, anti-inflammatory agents, and analgesics.[148] Percutaneous exposures necessitate judicious use of intravenous fluids and monitoring for symptoms associated with VLS.

Development of Ricin Small Molecule Inhibitors

Reaching intracellular space with a ricin inhibitor provides an ideal pre- and postexposure therapeutic. At a minimum, small molecule inhibitors must possess sufficient safety and efficacy to enable a pathway to licensure. A strong safety profile is critical since no diagnostic capability exists to identify personnel who have received a clinically significant dose of ricin. Ideally, the inhibitor is also self-administered, which would greatly reduce the burden on the healthcare system and allow the provider to focus on patients who require more intensive care and medical resources.

A variety of approaches have been used to identify suitable small molecule ricin therapeutics. Potential compounds fall into three broad mechanisms of action:

1. those that target RTA;
2. those that target the retrograde transport pathway used by ricin to gain access to the cytosol; and
3. a group that alters the cellular stress response following ricin intoxication.

A notable absence among published work includes molecules directed against the RTB that might prevent ricin from entering cells. However, the molecular

structure of RTB makes it an extremely difficult drug target. X-ray studies show that RTB is composed of two domains with each domain possessing three subdomains that bind to sugars.[62,149] Selected mutations of RTB suggest that three of the six sites must be inactivated to prevent cellular intoxication.[62] Since these three sites are widely separated on RTB, it would present a formidable challenge in the design of inhibitors that still possess drug-like characteristics. Furthermore, because the RTB carbohydrate binding regions are small and shallow, these features present yet another hurdle for the design of drug-like molecules.[150]

In contrast, RTA presents a more tractable drug target. Although the large, open, and polar nature of the active site makes it a difficult drug target,[151,152] high-resolution X-ray structures of the active site can help in the design of inhibitors.[153] Furthermore, the mechanism of action for ricin is well described and provides additional criteria for the design of drugs that target the active site. Drug discovery approaches for ricin therapeutics have typically relied on virtual screening (VS), or high throughput cell-based assays. Virtual screening uses computational methods to evaluate large numbers of compounds for possible activity against ricin but requires careful consideration of molecular parameters to ensure optimal results, access to libraries of appropriate chemicals,[154,155] and structural data, such as high resolution crystal structures of the target molecule.[156] No single VS software is ideal as each produces different results.[160] Several excellent recent in-depth reviews provide additional background on VS.[157–159] Although it allows for evaluation of a large number of chemicals, VS has not always identified appropriate candidates for drug development.[152,156,160] Regions of proteins, such as the active site of ricin, that have large and polar pockets are difficult drug targets,[151] but successes have been seen in discovering RTA inhibitors.[161,162,163]

Another screening technique, cell-based high throughput screening (HTS), requires an appropriate cellular model of intoxication and a method to identify compounds that have activity against ricin. For cell-based assays the tested compounds should be soluble in cell culture media or with an excipient compatible with cellular growth. The solubility requirement significantly reduces the number of compounds that can be tested in cell-based assays. Furthermore, poor solubility may mask an otherwise useful molecule because it cannot be delivered to the cells in a high enough concentration to have an observable effect. More comprehensive discussion on cell-based HTS can be found in several recent reviews.[164–167] Despite these limitations, the complementary methods of VS and cell-based HTS assays have identified a variety of active compounds. Similar approaches have been used to identify small molecule inhibitors of shigatoxin, a prokaryotic enzyme with related enzymatic activity but limited structural homology to ricin, suggesting pharmacophore discovery is broadly applicable.[151–161,168–170] Some of these VS and HTS successes for RTA inhibitor design will be highlighted in the next sections.

Ricin A Chain Inhibitors

Several research groups used RTA as a target to identify potential lead compounds from chemical libraries. One of the most potent active site inhibitors was developed by a rational drug design process[171] and built on an earlier observation that pteroic acid (PTA) bound to the ricin active site with a modest IC_{50} of 600 μm.[163] Although PTA is not a suitable candidate because of its limited solubility, it served as a platform for designing derivatives. Several derivatives of PTA were found to have increased solubility, and when a linker was included that enabled additional contacts between RTA and the inhibitor, RTA inhibition was enhanced.[172] Saito et al[171] built on this observation by adding di- and tri-peptide linkers to PTA that allowed binding both the specificity pocket and a distant secondary pocket within the ricin active site. The addition of these linkers and the resulting interaction with the secondary pocket provided a 100-fold improvement in the IC_{50}.[171]

Additional successes in identifying RTA inhibitors through virtual screening have been reported by Pang et al[161] and Bai et al.[162] Although both groups' compounds target RTA, Pang's[161] molecules targeted a site distant from the active cleft yielding inhibition of ricin enzymatic activity, whereas Bai's[162] are active site inhibitors. Pang's[161] deliberate choice was based on the recognition that the large size and polar features as well as the multiple electrostatic interactions between rRNA and the active site made it an unattractive and difficult drug target. Pang et al[161] also took advantage of a structural change that occurs in the ricin active site upon binding of the toxin to the α-sarcin-ricin loop in the 28S ribosome, which causes Tyr80 in the active cleft to move to a new position where it participates in the depurination of the ribosome by packing with the bases of rRNA.[153,173,174] Thus, if the movement of Tyr80 is blocked ricin is rendered inactive. Preventing the movement of the Tyr80 is a novel approach to developing RTA inhibitors by avoiding the complication of designing drugs for the ricin active site. This approach to inhibitor design was designated as the "door-stop" approach because it prevents Tyr80 from undergoing the necessary conformational change for enzymatic activity. Pang et al[161] screened more than 200,000 molecules with molecular weights lower than 300 Da and 226 were predicted to block the movement of Tyr80. When evaluated in a CFT assay using firefly

luciferase, several of these compounds inhibited ricin. Unexpectedly, several compounds enhanced the firefly luciferase assay, but were the result of the compounds directly interacting with the firefly luciferase and not RTA. This interaction precluded the determination of the IC_{50} of the compounds and serves to underscore that appropriate controls need to be present when screening chemicals for activity in this reporter assay.[175–178] However, functional studies revealed that the Pang compounds[151] protected cells exposed to ricin, suggesting that ricin inhibition using the "door-stop" approach is a validated model. Furthermore, these results demonstrated that direct competition with the ricin active site, a difficult target, was not essential to achieve inhibition of the ricin catalytic activity.

The VS approach conducted by Bai et al[162] identified several new classes of inhibitors. Bai[162] used two different VS programs, one to identify molecules that could bind to RTA in which the Tyr80 has been displaced and a separate program that identified candidate compounds that bound to the RTA form in which Tyr80 was not displaced. Compounds ranked highly by both programs were selected for further study, and they revealed a variety of new chemical entities for further development.[162] In vitro kinetic studies showed that these compounds possess a potency similar to PTA. Although many of the compounds were cytotoxic, two were identified that protected vero cells exposed to ricin. The best performing compound showed little cytotoxicity and protected about 90% of cells exposed to ricin.[162] Nevertheless, the cytotoxic compounds can still serve as starting points to improve their binding to RTA while reducing their toxicity.

Transport Inhibitors

The second category of inhibitors, transport inhibitors, blocks the retrograde movement of ricin through the cell and may have its greatest utility as preexposure treatments. Compounds that inhibit the retrograde transport of ricin have substantial efficacy in animal models when used in a preexposure setting.[179] However, because of the retrograde pathway taken by ricin to arrive at its cellular target, inhibitors of this normal cellular process also have a potential to exhibit significant toxicity. For example, ilimaquinone (IQ), a marine sponge metabolite, inhibited ricin in a dose-dependent manner in a vero cell assay.[180] However, IQ also caused the Golgi apparatus to fragment into smaller vesicles; yet this effect was reversible when IQ was removed.[181] Additional molecules have been identified that alter retrograde transport and protect cells from ricin challenge; however, the utility of these molecules for continued development is questionable because they also disrupt the Golgi architecture.[182] In spite of the potential toxicity of retrograde transport inhibitors, several groups identified inhibitors of ricin transport, some of which show limited toxicity in cellular and animal based assays of efficacy. Stechmann and colleagues[179] used a protein synthesis cell-based HTS assay to identify compounds that restored normal levels of protein synthesis after ricin exposure. Of more than 16,000 compounds, they identified two that were inhibitors of retrograde transport. Despite functionally blocking retrograde movement, these compounds exhibited no effect on the architecture of the Golgi complex or on cellular transport pathways such as endocytosis, vesicle recycling, degradation, or secretion.[179] These two compounds were further examined in an animal model of intranasal ricin challenge. The compounds completely protected challenged animals when treatment was given 1 hour before ricin exposure; no acute toxicity was observed in animals that received only the test compounds.[179] However, these compounds may not be ideal candidates for further development because of instability.[183]

Cellular Stress Response Inhibitors

Rather than targeting the ricin molecule or the retrograde transport pathway described previously, another target is the cellular response to ricin. When ricin depurinates ribosomes in target cells, these cells enter a condition known as ribotoxic stress response.[184,185] The ribotoxic stress response leads to activation of stress associated protein kinases and other cellular changes.[184] Activation of stress associated protein kinases including p38 mitogen activated protein kinase ($p38^{MAPK}$) can lead to the release of proinflammatory cytokines and the induction of apoptosis in cells.[186–188] A screen of molecules that protected cells from ricin challenge but did not act on ricin or the retrograde pathway identified two molecules for further analysis.[189] One compound reduced the activation of the SAPK $p38^{MAPK}$ by acting upstream of $p38^{MAPK}$ activation. The other compound acted as an inhibitor of caspase 3 and 7 activation, thus blocking a critical step in the induction of apoptosis.[189]

SUMMARY

Ricin is a potent toxin derived from the castor plant, *R communis L*, which has been cultivated worldwide for its oil since ancient times. Because of its potency, stability, wide availability of its source plants, and popularity on the Internet, ricin is considered a significant biological warfare or terrorism threat. Ricin was developed as an aerosol biological weapon during World War II, but was not used in combat nor in

mass casualty attacks. As a biological weapon, ricin has not been considered as useful in comparison with other biological agents such as anthrax or botulinum neurotoxin. Nevertheless, its popularity and its track record in actually being exploited by extremist groups and individuals accentuate the need to be vigilant of its surreptitious misuse.

Despite ricin's notoriety as a potential biological agent, its medical applications have been also explored. Ricin has contributed to early immunology; the understanding of both immunological and cell biological processes; and the treatment of cancer, AIDS, and other illnesses. Clinical manifestations of ricin poisoning vary depending on the routes of exposure. Aerosol exposure represents the greatest threat posed by ricin and can lead to death via hypoxia. Diagnosis of ricin exposure is based on both epidemiological and clinical parameters. No Food and Drug Administration-approved drug or vaccine against ricin intoxication exists; treatment is mainly symptomatic and supportive. Since vaccination offers a practical prophylactic strategy against ricin exposure, considerable efforts have been devoted to develop a safe and effective ricin vaccine to protect humans, in particular soldiers and first responders. Recombinant candidate ricin vaccines are currently in advanced development in clinical trials. Efforts are also underway to develop small molecule inhibitors for the treatment of ricin intoxication. Recent findings suggest that refinement of the newly identified ricin inhibitors will yield improved compounds suitable for continued evaluation in clinical trials.

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Chapter 17

STAPHYLOCOCCAL ENTEROTOXIN B AND RELATED TOXINS PRODUCED BY *STAPHYLOCOCCUS AUREUS* AND *STREPTOCOCCUS PYOGENES*

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INTRODUCTION

Staphylococcus aureus and *Streptococcus pyogenes* are ubiquitous, gram-positive cocci that play an important role in numerous human illnesses such as food poisoning, pharyngitis, toxic shock, autoimmune diseases, and skin and soft tissue infections. These common bacteria readily colonize humans via numerous virulence factors that facilitate their survival and dissemination. Among these factors, staphylococcal enterotoxins (SEs), toxic shock syndrome toxin-1 (TSST-1), and streptococcal pyrogenic exotoxins (SPEs) share a common three-dimensional protein fold characteristic of these bacterial exotoxins called "superantigens" due to their potency in activating cells of the immune system.[1,2] Picomolar concentrations of these bacterial superantigens activate specific Vβ-bearing T cells by binding to and cross-linking the major histocompatibility complex (MHC) class II molecules on antigen-presenting cells (APC) and the T-cell receptor (TCR). Activated T cells proliferate and, together with APC, produce proinflammatory mediators that, in elevated quantities, can induce fever, hypotension, and lethal shock. Most strains of *S aureus* and *S pyogenes* examined harbor genes for superantigens and are likely to produce at least one of these toxins. Strains that lack the ability to produce superantigens are usually attenuated in virulence. The staphylococcal enterotoxins are most frequently associated with food poisoning, yet not all superantigens are enterotoxins. Life-threatening toxic shock syndrome (TSS) may result from exposure to any of the superantigens through a nonenteric route. High dose, microgram-level exposures to staphylococcal enterotoxin B (SEB) will result in fatalities, and inhalation exposure to nanogram or lower levels may be severely incapacitating as well as fatal.[3] In addition, the severe perturbation of the immune system caused by superantigen exposure may lower the infectious or lethal dose of replicating agents, such as influenza virus.[4]

SEB is a prototype enterotoxin and potential biological threat agent produced by many isolates of *S aureus*. During the 1960s, SEB was studied extensively as a biological incapacitant in the US offensive program. Recent studies on countermeasures and diagnostics have focused on SEB because of its effectiveness as a biological weapon, especially by inhalation. However, SEB represents many related biologically active superantigens that are readily isolated and manipulated by recombinant DNA (deoxyribonucleic acid) techniques. Moreover, the coadministration of SEB or related toxins with replicating pathogens or pathogen-associated molecules can lower the lethal dose of toxin by thousands fold. Pathogen-associated molecules such as endotoxins bind to toll-like receptors (TLRs) present on many cell types and activate similar intracellular signaling pathways as SEB, accounting for the synergy between these molecules and SEB in inducing pathophysiological effects.[5]

CHARACTERIZATION OF TOXINS

Genes encoding superantigens of *S aureus* and *S pyogenes* arise from a common ancestral gene. Most of the streptococcal superantigens are encoded by mobile genetic elements. SPE-A, SPE-C, SEA, and SEE are all phage-borne, while SED is plasmid-encoded. A chromosomal cluster of SE and SE-like genes are present in strains of *S aureus*.[6] Transcriptional control of TSST-1, SEB, SEC, and SED is mediated through the accessory gene regulator (agr) locus,[7] whereas SEA expression appears to be independent of agr. Strains that are agr-negative generally produce less toxin; however, there are also considerable differences in production levels among agr-positive isolates. These toxins are synthesized during the late logarithmic to stationary phases of growth, and production of many SEs is dependent on glucose concentration and environmental pH. The great diversity of superantigens and the highly mobile nature of their genetic elements suggest an accelerated rate of evolution. Staphylococcal and streptococcal strains that colonize domestic animals are potential genetic reservoirs for new toxin genes,[8] and the transfer of these sequences may contribute to hybrid polypeptides.

The bacterial superantigens are 19- to 30-kD single-chain proteins with two major domains, containing β-sheet and α-helix structures, separated by a shallow groove.[1,9] Based on amino acid sequences and structural homology, superantigens can be compiled into five different groups.[10] TSST-1 is the most distantly related and lacks a "disulfide loop" commonly found in SEs, whereas SEs with emetic properties such as SEA, SEB, SEC, SED, and SEE all possess this loop structure. Despite significant sequence divergence, with similarities as low as 14%, overall protein folds are similar among staphylococcal and streptococcal superantigens. Cross-reactivities of polyclonal and monoclonal antibodies to SEs, TSST-1, and SPEs indicate common epitopes among these superantigens.[11] The toxin genes have evolved by strong selective pressures to maintain receptor-binding surfaces by preserving three-dimensional protein structure. The contact surfaces with MHC class II molecules involve variations of conserved structural elements,[12,13] which include a ubiquitous hydrophobic surface loop, a polar-binding pocket present in most superantigens,

and one or more zinc-binding sites found in some toxins. Comparison of antibody recognition among superantigens[11] suggests that antigenic variation is maximized while three-dimensional structures, and hence receptor-binding surfaces, are conserved. From a practical standpoint, this observation indicates that a large panel of antibody probes will be required for proper sample identification.

Molecular details of the receptor interaction and biological actions of bacterial superantigens are well established. Superantigens target cells that mediate innate and adaptive immunity, resulting in an intense activation and subsequent pathology associated with aberrant host-immune responses. In contrast to "conventional" antigens, bacterial superantigens bind on the outside of the peptide-binding groove of MHC class II molecules and exert their biological effects without being "processed." Most superantigens share a common mode for binding MHC class II molecules, with additional stabilizing interactions that are unique to each toxin.[14] A second, zinc-dependent molecular binding mode for some superantigens increases T cell signaling and may impart greater toxicities in some cases. In normal T-cell responses to peptide antigens, the CD4 molecule stabilizes interactions between TCR and MHC class II molecules on APC (Figure 17-1). Superantigens also cross-link TCR and MHC class II molecules, mimicking the CD4 molecule,[15] and hence stimulate large numbers of T cells. Recognition of a superantigen by TCR is dependent on the variable region of the β chain (Vβ) of the TCR. Each toxin binds to a distinct repertoire of TCR Vβ, thus revealing the unique Vβ specificities of an individual superantigen.[16] An intense and rapid release of cytokines, such as interferon-γ, interleukin-1 (IL-1), interleukin-2 (IL-2), interleukin-6 (IL-6), and tumor necrosis factor-α (TNFα) is responsible for the systemic effects of the toxins.[17–19] Although SEB has enterotoxic effects, the interaction of toxin with specific cells and receptors of the gastrointestinal tract is less well-defined. A specific region in SEB is involved in transcytosis of toxin.[20] Other studies suggest various binding regions of SEB to epithelial cell membrane proteins.[21,22] The release of histamine and cysteinyl leukotriene from mast cells likely accounts for the emetic effects of staphylococcal enterotoxins.[23]

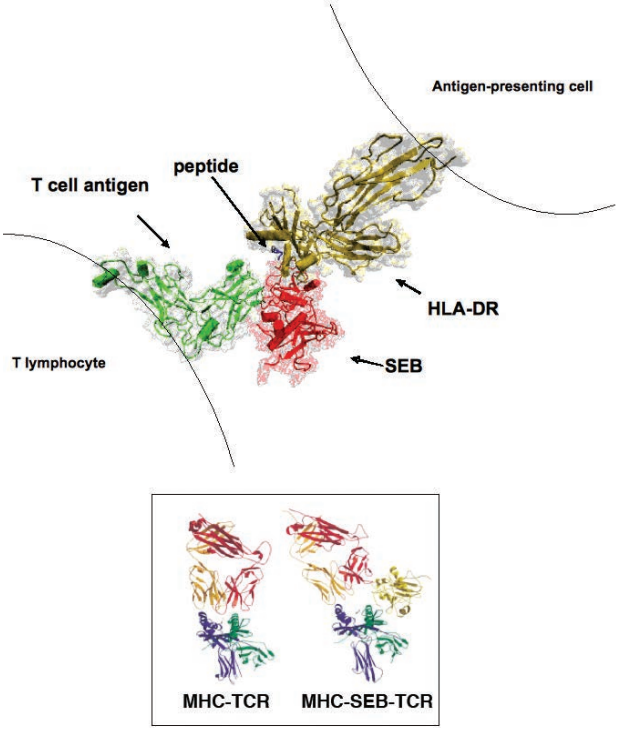


Figure 17-1. Molecular model of receptor binding. Staphylococcal enterotoxins and other bacterial superantigens target the multireceptor communication between T cells and antigen-presenting cells that is fundamental to initiating pathogen-specific immune clearance. The superantigen inserts itself between the antigen receptor of T cells and the major histocompatibility complex class II molecule displaying peptides from potential pathogens. Toxin exposure results in hyperactivation of the immune system, and the pathology is mediated by tumor necrosis factor-α, interferon-γ, and other cytokines.
HLA-DR: Human Leukocyte Antigen DR; SEB: staphylococcal enterotoxin B; TCR: T-cell receptor

HOST RESPONSE AND ANIMAL MODELS

Individuals may respond differently to superantigen exposure as a result of MHC polymorphisms, age, and many physiological factors. Each toxin exhibits varying affinities toward the HLA-DR, HLA-DQ, and HLA-DP isotypes and distinct alleles of class II MHC molecules, as observed by differences in T-cell responses in vitro. Generally, SE and TSST-1 bind HLA-DR better than HLA-DP or -DQ, whereas SPEA preferentially binds HLA-DQ better than HLA-DR. Primates, including humans, are most sensitive to superantigens when compared to other mammals.[24] Lethal or incapacitating doses of toxin may be lowered by coexposure to endotoxin from gram-negative bacteria[17] or hepatotoxins,[25] or by infection with replicating agents.[4]

At the cellular level, the interaction of superantigens with receptors on APC and T cells leads to intracellular signaling.[26] As with conventional antigens, costimulatory receptors are also required for cell activation by superantigens. The best-characterized costimulatory receptors are CD80/CD86 on APC and CD28 on T cells.[27–29]

The expression of intercellular adhesion molecule-1 (ICAM-1) on APC promotes stable cell conjugate with T cells and provides costimulatory activation signals.[27] The interactions of LFA-1 (lymphocyte function-associated antigen)/ICAM-1 and CD28/CD80 have both been implicated in SEA (staphylococcal enterotoxin A)-mediated T-cell activation.[30] High concentrations of SEB elicit induction of phosphatidylinositol and the activation of protein kinase C (PKC) and protein tyrosine kinase (PTK) pathways,[31,32] similar to mitogenic activation of T cells. PKC and PTK activation affect many intracellular signaling pathways, ultimately activating the transcription factors NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), NF-AT (nuclear factor of activated T cells), and AP-1 (activator protein 1), resulting in the expression of proinflammatory cytokines, chemokines, and adhesion molecules.[33–35] Both IL-1 and TNFα can directly activate the transcription factor NF-κB in many cell types, including epithelial cells and endothelial cells, perpetuating the inflammatory response. Another mediator, IFNγ (interferon gamma), produced by activated T cells and natural killer cells, synergizes with TNFα and IL-1 to enhance immune reactions and promote tissue injury. PTKs and T-cell cytokines also activate phosphoinositide 3 kinase (PI3K), affecting many intracellular processes and pathways, ultimately activating the mammalian target of rapamycin (mTOR).[36] SEB and other superantigens also directly induce chemotactic mediators, interleukin-8, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1α, and macrophage inflammatory protein-1β, which can selectively chemoattract and activate leukocytes.[37–39] Thus, cellular activation by SEB and other superantigens leads to severe inflammation, hypotension, and shock. Additional mediators contributing to SEB-induced shock include prostanoids, leukotrienes, and tissue factor from monocytes; superoxide and proteolytic enzymes from neutrophils; and chemokines from epithelial and endothelial cells. Activation of coagulation via tissue factor leads to disseminated intravascular coagulation, tissue injury, and multiorgan failure. SE-induced TSS thus presents a spectrum and progression of clinical symptoms, including fever, tachycardia, hypotension, multiorgan failure, disseminated intravascular coagulation, and shock.[40,41]

In humans and nonhuman primates (NHP), SEs induce an emetic response and toxic shock when ingested.[42,43] Typically, the SEB-intoxicated NHPs developed anorexia, vomiting, and diarrhea within 6 to 24 hours postexposure, followed by depression, dyspnea, and shock 24 to 72 hours later.[43,44] Specific cells and receptors in the intestinal tract have not been identified for emesis, but some studies suggest the interaction of a dodecapeptide binding region of superantigen with epithelial cells.[21,22] Pulmonary edema and lung lesions with infiltrated leukocytes and macrophages appeared in NHP exposed to SEB.[44]

Although the SE studies in NHP are considered a "gold standard" for in vivo investigations, many rodent models have been developed as alternatives to study the toxic shock and acute lung injury aspects of superantigens.[4,17,25,37,39,45] The lower cost associated with maintaining mice, the availability of immunological reagents, and certain similarities to NHP models are obvious advantages for their use in the development of therapeutics and vaccines.

Mice are naturally less susceptible to SEs, TSST-1, and SPEs because of the lower toxin affinity to murine MHC class II molecules.[17] Potentiating agents, such as D-galactosamine,[25] actinomycin D,[46] lipopolysaccharide (LPS),[17] and viruses[4] have often been used to amplify the toxic effects of superantigens so that lower, practical amounts of toxins can be used for in-vivo studies. Many vaccine studies with SEB have been accomplished with an LPS-potentiated mouse model, as a natural synergy exists between these bacterial exotoxins and LPS.[17,47] Results of these studies show a correlation between increased serum levels of IL-1, IL-2, TNFα, and IFNγ with bacterial superantigen-induced shock. Pulmonary lesions with severe interstitial and alveolar edema, as well as perivascular leukocytic infiltrates in mouse models were similar to those in NHP exposed to SEB.[45,46] Transgenic mice with inserted human HLA class II molecules have also been developed to study SEB-induced shock.[48–50] In some cases, high doses of SEB and D-galactosamine were still required to induce toxic shock with transgenics.[48,50] Nevertheless, there is a correlation of proinflammatory cytokine induction and pulmonary lesions in the various transgenic models of SEB-induced toxic shock. A "double-hit" low-dose SEB model was developed in C3H/HeJ mice, an LPS-resistant mouse strain, to simulate human SEB-induced toxic shock.[39] This model mimics human TSS closely as intranasal delivery of SEB triggers lung inflammation, systemic release of cytokines, and hypothermia that culminate in death at later time points similar to human toxic shock.[45] All of these murine models have various drawbacks but they are useful as tools for the development of therapeutics and vaccines.

CLINICAL DISEASE

The clinical documentation of TSS provides the most comprehensive source of information on the pathology of superantigen (eg, SEB) exposure. To meet strict criteria of the Centers for Disease Control and Prevention for TSS,[51] negative blood (except for *S aureus* or *S pyogenes*), throat, or cerebrospinal

fluid cultures, as well as negative serological tests for Rocky Mountain spotted fever, leptospirosis, and measles should be obtained. TSS disease symptoms are characterized by a rapid drop in blood pressure, elevated temperature, and multiple organ failure. The profound hypotension and desquamation of the palms and soles of the feet characteristic of TSS is not observed in exposure by inhalation, and respiratory involvement is rapid, unlike other forms of TSS. Furthermore, the fever prominent after aerosol exposure is generally not observed in cases of SEB ingestion.

An accidental laboratory inhalation exposure of nine laboratory workers to SEB best exemplifies the clinical disease as reported below. The following description illustrates a severely incapacitating illness of rapid onset (3–4 hours) and modest acute duration (3–4 days) upon exposure to SEB.[43] Details of the disease and signs and symptoms are described below.

Fever

Fever was prominent in all nine of those exposed. Eight of the individuals experienced at least one shaking chill that heralded the onset of illness. Using the morning peak level of SEB aerosol generation in the laboratory as the most likely time of exposure, onset of fever occurred from 8 to 20 hours post initial exposure, with a mean time of onset of 12.4 ± 3.9 (SD) hours. Duration of fever was from 12 to 76 hours after onset, with a mean duration of 50 ± 22.3 hours. Fever ranged as high as 106° acutely. Myalgias were often associated with the initial fever. Onset of myalgia was between 8 and 20 hours, with a mean onset of 13 ± 5 hours. Duration was from 4 to 44 hours, and the mean duration was 16 ± 15 hours.

Respiratory Symptoms

All nine patients were admitted to the hospital with a generally nonproductive cough. Onset was at 10.4 ± 5.4 hours, and duration was 92 ± 41 hours. Five had inspiratory rales with dyspnea. The three most seriously compromised patients had dyspnea, moist inspiratory and expiratory rales, and orthopnea that gradually cleared. One individual had profound dyspnea for the first 12 hours that moderated to exertional dyspnea and rales, which persisted for 10 days. Chest radiographs on admission showed densities compatible with "patches of pulmonary edema" and Kerley lines suggesting interstitial edema. During recovery, discoid atelectasis was noted. Moderate compromise of the respiratory system was often accompanied by radiographic evidence of peribronchial accentuation, or "cuffing." The mildly ill patients had normal radiographs. One of the three severely ill patients had severe pulmonary compromise and profound dyspnea and received only slight relief when treated with an aminophylline suppository. Moderately intense chest pain, of a substernal pleuritic type, occurred in seven individuals. Onset of chest pain was at 12 ± 6.5 hours and lasted for 4 to 84 hours, with a mean duration of 23 ± 27 hours.

Headache

Eight of the nine patients experienced headache with onset ranging from 4 to 36 hours, and the mean time of onset was at 13.3 ± 10 hours. Duration ranged from 8 to 60 hours, with a mean duration of 30.6 ± 19 hours. The headaches ranged from severe to mild, but were usually mild by the second day of hospitalization. Five individuals' headaches responded to Darvon (propoxyphene hydrochloride; Eli Lilly & Company, Indianapolis, IN) or codeine.

Nausea and Vomiting

Gastrointestinal symptoms occurred in more than half of the individuals, nausea and anorexia in six, and vomiting in four. The onset of nausea ranged from 8 to 24 hours, with a mean onset of 17 ± 6.3 hours. Duration ranged from 4 to 20 hours, with a mean of 9 ± 5.5 hours. The time to onset of anorexia ranged from 8 to 24 hours, with a mean onset of 18.5 ± 5.6 hours. Duration of anorexia ranged from 4 to 136 hours, and the mean duration was 44.5 ± 45 hours. Vomiting occurred in four patients, sometimes after prolonged paroxysms of coughing. The range of onset of vomiting was 8 to 20 hours, with a mean time to onset of 14 ± 5.1 hours. Duration was not prolonged and usually consisted of one episode. The patients were successfully treated with Compazine (prochlorperazine; SmithKline Beecham Pharmaceuticals, Philadelphia, PA) and Benadryl (diphenhydramine hydrochloride; Pfizer Pharmaceuticals Company, New York, NY). Only one individual demonstrated hepatomegaly and bile in the urine, although another patient also demonstrated mildly elevated liver-function tests. No diarrhea was reported in any of the exposed individuals.

Other Signs and Symptoms

Cardiovascular

All patients who experienced chest pain had normal electrocardiograms. Throughout the illness, all patients were normotensive. Vomiting was of brief duration, and no one, including those vomiting, required

intravenous fluid administration. The patients' pulse rates, when elevated, paralleled temperature elevation.

Hematology

Leukocytosis was observed in most of the patients 12 to 24 hours after exposure to the toxin.

Ocular Effects

None of the patients experienced conjunctivitis, although one individual later stated he remembered that his eyes had "burned" during the believed time of exposure. This contrasts with reports of conjunctivitis resulting from separate accidental laboratory exposures.[52]

DETECTION AND DIAGNOSIS

The staphylococcal enterotoxins are moderately stable proteins; therefore, immunological evaluation should be possible in field or clinical samples. A variety of rapid and sensitive detection methods are available.[53,54] Immunoassays are very sensitive and can detect picogram quantities of toxins in environmental and serum samples. Plasma concentrations of superantigens were measured in septic patients of an intensive care unit using an enzyme-linked immunosorbent assay.[55] In one study, [56] the mean concentration of TSST-1 in human sera from TSS patients was reported to be 440 pg/mL. In contrast, anti-TSST-1 antibody titers are often low in TSS patients[57,58] and only recover during convalescence. Furthermore, most normal human serum samples contain detectable levels of antibody reacting with several different toxins, including SEB. Therefore, serum antibody titers are of little diagnostic value. If bacterial sepsis is suspected and cultures can be obtained, detecting minute quantities of potentially toxigenic strains is possible using polymerase chain reaction (PCR) amplification and toxin gene-specific oligonucleotide primers. The results from both PCR and immunoassays are rapid, allowing quantitative or qualitative measurements in less than 24 hours. Finally, as the best approach to early diagnosis on the battlefield, toxins may be identifiable in nasal swabs from individuals exposed to aerosols for at least 12 to 24 hours postexposure.

MEDICAL MANAGEMENT

No specific therapy has been identified or described.[3,41,43] Supportive therapy in the nine mild accidental exposure cases described earlier seemed to provide adequate care. Symptoms of fever, muscle aches, and arthralgias may respond to cool compresses, fluids, rest, and judicious use of acetaminophen or aspirin. For nausea, vomiting, and anorexia, symptomatic therapy should be considered.

Antihistamines (eg, diphenhydramine) and phenothiazine derivatives (eg, prochlorperazine) have been used parenterally or as suppositories. The success of these drugs in controlling nausea may have been augmented by the relatively short duration of nausea and vomiting induced by aerosolized SEB. Because of the brevity of vomiting episodes, fluid replacement was not considered or required in the series discussed. However, replacement may be necessary in the event of prolonged vomiting resulting in fluid and electrolyte depletion. Although diarrhea was not observed in human accidental exposure cases, deposition of toxin on foodstuffs could produce the syndrome, which should be treated symptomatically.

Initial symptomatic therapy with cough suppressants containing dextromethorphan or codeine should be routinely employed. Prolonged coughing unrelieved by codeine might benefit from a semisynthetic, centrally acting narcotic antitussive containing hydrocodone (dihydrocodeinone).

Pulmonary status should be monitored by pulse oximetry, and when respiratory status is compromised, prompt evacuation to a site with capacity for intensive respiratory care by mechanical ventilation should be considered.

Infusion of intravenous immunoglobulin has been successfully used[59,60] to treat episodes of Kawasaki's syndrome linked to SE and TSST-1. An anecdotal case of TSS with elevated TSST-1 and SEA levels, complicated by life-threatening multiorgan dysfunction, was successfully treated by early introduction of plasma exchanges.[61] Prior exposure to SEB by inhalation does not appear to protect against a subsequent episode; however, increased antibody titers to SEB are protective, and efforts to devise both passive and active immunotherapy show promise. Because of the rapidity of receptor binding by these toxins (apparent saturation less than 5 minutes), active immunity should be considered the best defense.

The treatment of toxic shock with *S aureus*-secreting superantigens such as SEB and TSST-1 is much more complex in a clinical setting. Both *S aureus* and *S pyogenes* produce multiple virulence factors that aid in bacterial survival and dissemination in the host.

Furthermore, the emergence of methicillin-resistant *S aureus* strains poses constraint in treatment options and clinical guidelines were revised and updated recently.[62] A recent study in a rabbit model of *S aureus* pneumonia suggests that vaccination against superantigens and secreted cytolysins provides protection against *S aureus*, whereas vaccination against bacterial cell-surface antigens increases disease severity.[63]

VACCINES

A formalin-treated SEB toxoid demonstrated some degree of efficacy in animal trials, but is not approved for human use. Vaccines produced by site-specific mutagenesis of the toxins, delivered by intramuscular or intradermal routes, have also shown promising results in animal and human trials. These recombinant subunit vaccines were produced by substitution of active receptor-binding amino acid side chains that reduced affinities and consequential T-cell activation[13,14,47,64] without altering the three-dimensional structure of the antigen. Though promising, these engineered vaccines are neither licensed nor available for human use.

DEVELOPMENT OF THERAPEUTICS

An understanding of the cellular receptors, signaling pathways used by staphylococcal superantigens, and the biological mediators induced has provided insights to selecting appropriate therapeutic targets. Potential targets to prevent the toxic effects of SEs include (*a*) blocking the interaction of SEs with the MHC, TCRs,[26] or other costimulatory molecules[27,28]; (*b*) inhibition of signal transduction pathways used by SEs[26]; (*c*) inhibition of cytokine and chemokine production[36]; and (*d*) inhibition of the downstream signaling pathways used by proinflammatory cytokines and chemokines.

Limited therapeutics for treating superantigen-induced toxic shock are currently available. Intravenous immunoglobulin was effective as a treatment in humans after the onset of toxic shock syndrome.[41,59] Antibody-based therapy targeting direct neutralization of SEB or other superantigens is most suitable during the early stages of exposure before cell activation and the release of proinflammatory cytokines.[64] Because some neutralizing antibodies cross-react among different superantigens,[11] a relatively small mixture of antibodies might be effective in treating exposures to a greater variety of superantigens. Vaccines of SEB and SEA with altered critical residues involved in binding class II MHC molecules were also used successfully to vaccinate mice and monkeys against SEB-induced disease.[47,65]

Most therapeutic strategies in animal models of SEB-induced shock have targeted proinflammatory mediators. Therapeutic regimens include corticosteroids and inhibitors of cytokines, caspases, or phosphodiesterases.[45,66,67] Several in vivo murine models have been used to study potential therapies that prevent superantigen-induced shock. Therapeutic agents, such as nitric oxide inhibitors, decrease SEA and SEB effects by inhibiting the production of IL-1, -2, -6, TNFα, and IFNγ in the LPS-potentiated model.[68] Blockade of the CD28 costimulatory receptor by its synthetic ligand, CTLA4-Ig, prevented TSST-1-induced proliferation of T cells and lethal TSS.[69] Decreased mortality rates accompanied by an attenuation in liver apoptosis and hemorrhagic necrosis were seen in mice given D-galactosamine plus SEB along with a cell-permeable cyclic peptide targeting NFκB.[70] Dexamethasone, a well-known FDA-approved immunosuppressant and NFκB inhibitor, prevented toxic shock in the LPS-potentiated mouse model and the "double-hit" SEB-induced shock model.[45,71] Rapamycin, another FDA-approved drug currently used to prevent kidney graft rejection, was efficacious even when given 24 hours after SEB in the "double-hit" SEB-induced shock model.[72] Recently, myeloid differentiation primary response protein (MyD88)–mediated proinflammatory signaling has been shown to be activated after SEB binding to MHC class II[73] and that MyD88$^{-/-}$ mice are resistant to SEB and SEA intoxication.[74,75] Administration of a synthetic small molecule mimetic (EM-163) to the conserved BB loop in the toll/IL-1 receptor (TIR) domain of MyD88, reduced multiple cytokines and protected mice from lethal shock in the LPS-sensitized model. [76,77]

SUMMARY

SEB is representative of a group of bacterial proteins that exert profound toxic effects upon the immune system. Many sensitive immunoassays have been developed for laboratory detection of most of the staphylococcal and streptococcal superantigen toxins, but the limit of field detection is unknown. Inhalation exposure to agents such as SEB may result in severe but temporary incapacitation, while high-dose exposures

will result in fatalities. Supportive symptomatic therapy is the only known method of treatment. Vaccines currently under development may afford protection to individuals but are not yet licensed for human use. Therapeutics tested in murine models may provide insights to future development in treating toxic shock.

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Chapter 18

TOXINS FROM VENOMS AND POISONS

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INTRODUCTION

SOME DEFINITIONS: VENOMS, TOXINS, AND POISONS

NONWARFARE EPIDEMIOLOGY OF VENOM-INDUCED DISEASES AND RELATED TOXINS
- **Venomous Bites and Stings**
- **Venomous Snakes**
- **Scorpions, Spiders, and Other Arachnids**
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MAJOR TOXIN CLASSES AND THEIR CLINICAL EFFECTS
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- **Nectrotoxins**
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CONCLUSIONS AND DIRECTIONS FOR RESEARCH

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INTRODUCTION

This chapter considers toxins that might be exploited as offensive biological weapons, or that may have medical relevance to deployed military personnel. The major characteristics of important toxin classes are summarized, and their medical effects are covered. Venom toxins are emphasized because little information is available about venomous animals in relation to military medicine. This chapter highlights selected plant, fungal, and animal toxins as examples of potent agents that target essential physiological processes, and it provides information that may facilitate recognition of types of envenoming or poisoning in an affected patient. This chapter also supports the perception that animal toxins are generally of low relevance to military applications, and contrarily, the relevant—but limited—nontactical importance of some plant and fungal toxins. This information is intended to increase awareness of the potential hazards posed by animal toxins that can be used for offensive applications on a small scale and also provide some important considerations about possible exposures to venomous and poisonous animals, plants, and mushrooms that might occur during military deployments.

The use in warfare of diverse animal-derived venoms, as well as the venomous animals themselves, has probably been contemplated for most of human history. The well-known mythical account of the second labor of Hercules slaying the malevolent nine-headed serpent, the Lernaean Hydra (Figure 18-1), featured him using venom-coated arrowheads to accomplish the deed. Some folklore scholars consider this to be the first description of the use of a bioweapon.[1] The practice may have been used in any of the ancient Greek Wars, and, as has been noted by numerous authors, the word toxic is derived from toxikon, Greek for poison arrow. This is one of the reasons why Findlay E Russell (1919–2012), in consultation with other founders of the International Society on Toxinology (IST), named the IST journal *Toxicon*. Circa 200 BCE, Hannibal reportedly used pottery containing venomous snakes to "bombard" opposing maritime vessels. The Roman Legion's assault on Hatra in 199 CE resulted in retaliation by civil forces that included the hurling of clay pots filled with scorpions over the walls.[1] Several Native American tribes (eg, Wishram, Yuma) used venom- or venom gland extract-coated arrowheads in warfare, and some such as the Achomawi and Karok used arrowheads dipped in rattlesnake organs or their extracts that they believed to be toxic.[2]

Historically, the offensive military use of venoms and their component toxins (all of natural origin) has been a rare and small-scaled occurrence. Venoms or venom-derived toxins have not generally been considered suitable for use as a mass offensive weapon; however, primarily fungal- or plant-derived toxins certainly have been weaponized (eg, ricin; see chapter 16). Using venoms as a weapon is also obviously distinct from the weaponization of toxins from bacteria.

However, many toxins found in animals, plants, and mushrooms are highly toxic—even lethal—to humans. These toxins, which can form the basis for developing tailored toxin derivatives for specific functions, are the subject of current intense research in the pharmaceutical industry. A well-known example is paclitaxel, a taxane initially derived from bark extract of the Western yew, *Taxus brevifolia*. Taxanes, such as paclitaxel, are potent cytotoxins that stabilize micro-



Figure 18-1. Hercules slaying the Lernaean Hydra. Illustration: Antonio del Pollaiolo, *Ercole e l'Idra e Ercole e Anteo*, Google Art Project. Wikimedia Commons, public domain. https://it.wikipedia.org/wiki/File:Antonio_del_Pollaiolo_-_Ercole_e_l%27Idra_e_Ercole_e_Anteo_-_Google_Art_Project.jpg.

tubule assembly, thereby disrupting physiological assembly/disassembly of microtubules in a guanosine triphosphate-independent manner.[3] These taxanes, which have a proven pharmacotherapeutic efficiency against a wide array of solid neoplasms, are a significant part of the chemotherapeutic armamentarium.

Several pharmacotherapeutics are derived from venom components, and some are in various stages of clinical trials. Two prominent examples are the entire class of antihypertensives: (1) the angiotensin-converting enzyme inhibitors (ACEIs) and (2) the parenteral insulin secretagogue exendin-4. Exendin-4 is a 39-amino acid peptide isolated from venom of the helodermatid lizard, the Gila monster (*Heloderma suspectum*), that has greater than 50% structural homology with glucagon-like peptide 1. Exendin-4 exhibits functional similarity with glucagon-like peptide 1, but has a longer half-life and biological stability. This peptide increases insulin secretion, accelerates gut emptying, and stimulates β-islet cell proliferation and survival, as well as other actions. It was tested as an antidiabetic agent and introduced as Byetta (Amylin Pharmaceuticals, San Diego, CA) in 2005.[4] Similarly, the ACEI arose from the study of bradykinin-potentiating oligopeptides present in several South American lance head pit vipers (eg, the jararaca, *Bothrops jararaca*, and others) in conjunction with study of the complex renin-angiotensin and kallikrein-kinin systems. Extensive investigation eventually resulted in teprotide, an early, parenterally administered ACEI, and eventually the first oral ACEI, Captopril (Par Pharmaceutical, Woodcliff Lake, NJ), was developed.[5] A broad variety of ACEIs have become among the three most frequently prescribed classes of antihypertensive medications in the United States and most of the world.

The potential threat posed by these toxins and their derivatives (as discussed further in the section on Relevance to Biological Warfare) relates mostly to their practicality for weaponization and delivery, rather than their inherent toxicity.

SOME DEFINITIONS: VENOMS, TOXINS, AND POISONS

Toxins are substances produced by living organisms (animals, plants, mushrooms, bacteria) that cause significant adverse effects when administered to another living organism, particularly those that offer the producing organism some advantage, either offensive or defensive.

Venoms are mixtures—often complex mixtures—of toxins produced in defined organs (usually venom glands) or organelles (eg, nematocysts/cnidocysts located in specialized cells and nematocytes/cnidocytes in jellyfish) that are delivered to the target organism usually using an evolved delivery system such as fangs or a stinging apparatus. Therefore, venom is delivered as an active process, and if sufficient (this may be minute in some cases) amounts are introduced into the target organism, it causes envenomation (also known as "envenoming"). Venom may be used defensively against predators (eg, stings from bees or venomous fish), but more commonly it is used offensively to assist in acquiring prey (eg, as by venomous snakes). Venom used offensively may be used to either kill or immobilize the prey, possibly aid digestion of the prey, or combine these functions, which may also be useful when venom is used defensively. The evolution of venom has been positively selected among a wide range of taxa, suggesting that it provides diverse organisms with selective advantages and fitness. The definition of venom from evolutionary, phylogenetic, and functional perspectives is actively debated.[6–10] The criteria defining the words venom and venomous and the related terminology may be subjected to interdisciplinary consensus in the future.[11]

Poisons are technically differentiated from venoms because they need to be ingested rather than injected (as venom is delivered) to induce their toxic effects. Animal, plant, or fungal toxins consist of individual or mixtures of toxins that are produced by the organism or, in some cases, by symbiotic bacteria (eg, the synthesis of tetrodotoxin by at least 18 microbial taxa, including *Vibrio* spp[12,13] and *Shewanella putrefaciens*[14]) that colonize the poisonous animal. These toxins are generally delivered in a passive and, in most cases, a defensive way to an organism attacking or trying to consume the toxin producer. Examples include tetrodotoxic fish that cause poisoning when eaten, some toads (eg, common African toad, *Amietophrynus regularis*) that exude toxins from parotid skin glands when mouthed by predators, and both poisonous plants and mushrooms when ingested. However, other animals such as several species of hedgehogs (eg, four-toed, spiny, or Cape hedgehog, *Atelerix albiventris pruneri*[15]) anoint their spines with toxic toad parotid secretions, and thereby can be considered to actively deter predators by exposing them to toad toxins via their spines. Therefore, in nature, poisoning by toxins is most often a passive process because the poison is introduced by the aggressor organism's actions. In terms of natural selection, it is often better to deter rather than kill a predator. When delivered at the typically delivered dose, the toxins in poisoning can often cause unpleasant but nonlethal effects. Clear exceptions exist when ingestion of only a tiny quantity of some of these poisonous organisms, because of their high lethal potency, can be fatal for humans.

NONWARFARE EPIDEMIOLOGY OF VENOM-INDUCED DISEASES AND RELATED TOXINS

Toxin-induced disease affects millions of humans every year. Detailed epidemiology is unavailable for any toxin-induced disease (other than selected microbial toxin diseases, and this information is often incomplete) at the global level, but more epidemiologic data may emerge for some key disease types, as a result of increasing international efforts directed at the improved management of regionally important venom diseases (eg, several snakebite initiatives). An approximate estimate of epidemiology for some principal groups is provided in Table 18-1.

Venomous Bites and Stings

Venomous animals include a vast array of organisms found in many phyla, from primitive to highly advanced, but certain groups have a particularly important impact on human health.

Venomous Snakes

Snakebite has the most significant impact on human health. Most regions contain some venomous species, but the rural tropics have a particularly high

TABLE 18-1

ESTIMATED HUMAN IMPACT OF ENVENOMING AND POISONING BY SOME PRINCIPAL GROUPS OF TOXIN-PRODUCING FAUNA AND FLORA*

	Estimated Annual Global Impact	
Organism Group	**Number of Cases**	**Number of Fatalities**
Venomous snakes	>2.5 million	>100,000
Scorpions	>1 million	>3,000
Spiders	>100,000	<100
Paralysis ticks	>1,000	<10
Insects†	>1 million	>1,000
Spiny venomous fish	>100,000	<10, likely close to zero‡
Stingrays	>100,000	<10§
Cone snails	<1,000	<10
Octopus (blue-ringed octopuses)	<100	<10
Jellyfish and related coelenterates	>1 million	<10¥
Fugu poisoning (tetrodotoxic fish)	Unknown	Unknown, likely >100
Ciguatera (ciguatoxic fish)	>20,000	Unknown, but few
Shellfish poisoning (several types)	Unknown	Unknown
Poisonous mushrooms	Unknown	Unknown, likely >100
Poisonous plants	Unknown	Unknown, possibly >1,000

*Data are based on most reliable published data, extrapolations of that data, or best guess estimates based on fragmented published data. The most reliable data are for snakebite and the annual global fatalities are probably underestimated. Most authorities consider published epidemiology data for envenoming as underestimates, and it is possible the estimated incidence given here is similarly an underestimate.
†Figures for insects include severe and fatal allergic reactions to venomous stings, which are responsible for the vast majority of medically significant cases.
‡No well-documented fatalities exist, and insufficient evidence for any approximations of possibly reliably reported semianecdotal cases.
§The handful of rare fatalities almost always results from intraperitoneal penetrative envenoming.
¥Almost all of the uncommon fatalities occur after envenoming by *Chironex fleckeri*, or one of several other chirodropid or charybdeid taxa that cause Irukandji syndrome (see Marine Envenoming in text).
Data sources: (1) Williams D, Gutiérrez JM, Harrison R, et al. The global snake bite initiative: an antidote for snake bite. *Lancet*. 2010;375:89–91. (2) Skinner MP, Brewer TD, Johnstone R, Fleming LE, Lewis RJ. Ciguatera fish poisoning in the Pacific Islands (1998 to 2008). *PLoS Negl Trop Dis*. 2011;5:e1416. doi:10.1371/journal.pntd.0001416. (3) Mebs D. *Venomous and Poisonous Animals: A Handbook for Biologists, Toxicologists and Toxinologists, Physicians and Pharmacists*. Boca Raton, FL: CRC Press; 2002: 360. (4) Meier J, White J. (eds). *Handbook of Clinical Toxicology of Animal Venoms and Poisons*. Boca Raton, FL: CRC Press; 1995. (5) Wang DZ. Neurotoxins from marine dinoflagellates: a brief review. *Mar Drugs*. 2008;11:349–371. (6) Noguchi T, Arawaka O. Tetrodotoxin-distribution and accumulation in aquatic organisms, and cases of human intoxication. *Mar Drugs*. 2008;6:220–242. (7) Warrell DA. Venomous bites, stings and poisoning. *Infect Dis Clin North Am*. 2012;26:207–223. (8) Personal observations of the authors.

burden of envenoming. The estimated global toll from snakebite remains a mixture of some quality evidence and projected speculation. Recent estimates suggest more than 2.5 million cases per year, with more than 1 million of these resulting in significant morbidity, approximately 400,000 cases requiring amputations, and more than 100,000 fatalities.[16] The economic impact likely is correspondingly enormous, but it has yet to be reliably quantified. Despite this impact, snakebite has generally been relegated to minor status in medical planning. The World Health Organization (WHO) briefly classified snakebite as one of three globally important "other neglected conditions" included under the recognized grouping of "neglected tropical diseases" that contains 17 infectious diseases responsible for a large proportion of morbidity and mortality in rural Third World regions.[17] However, in 2015, the WHO removed it from the list, and thus the considerable global impact of snakebite (especially among the world's most medically underserved communities) is no longer recognized (see http://www.who.int/gho/neglected_diseases/en/).

Scorpions, Spiders, and Other Arachnids

Scorpion stings are second, after snakebite in regards to medically significant occurrences, and probably affect more than 1 million humans each year, but with a low fatality rate (see Chippaux and Goyffon, 2010).

Spiderbite is also common, but with a few notable exceptions (widow spiders, *Latrodectus* spp, family *Theridiidae* [Figure 18-2]; recluse spiders, *Loxosceles* spp, family *Sicariidae* [Figure 18-3]; banana spiders, *Phoneutria* spp, family *Ctenidae*; Australian funnel-web spiders, *Atrax* spp [Figure 18-4]; and *Hadronyche* spp, family *Hexathelidae*), these are most commonly of minor medical significance (see www.toxinology.com). Tick envenoming causing paralysis is a problem in Australia, North America, and southern Africa, and possibly elsewhere, but reported cases are few, although rare fatalities have occurred (see Meier and White, 1995). The ticks involved are often members of

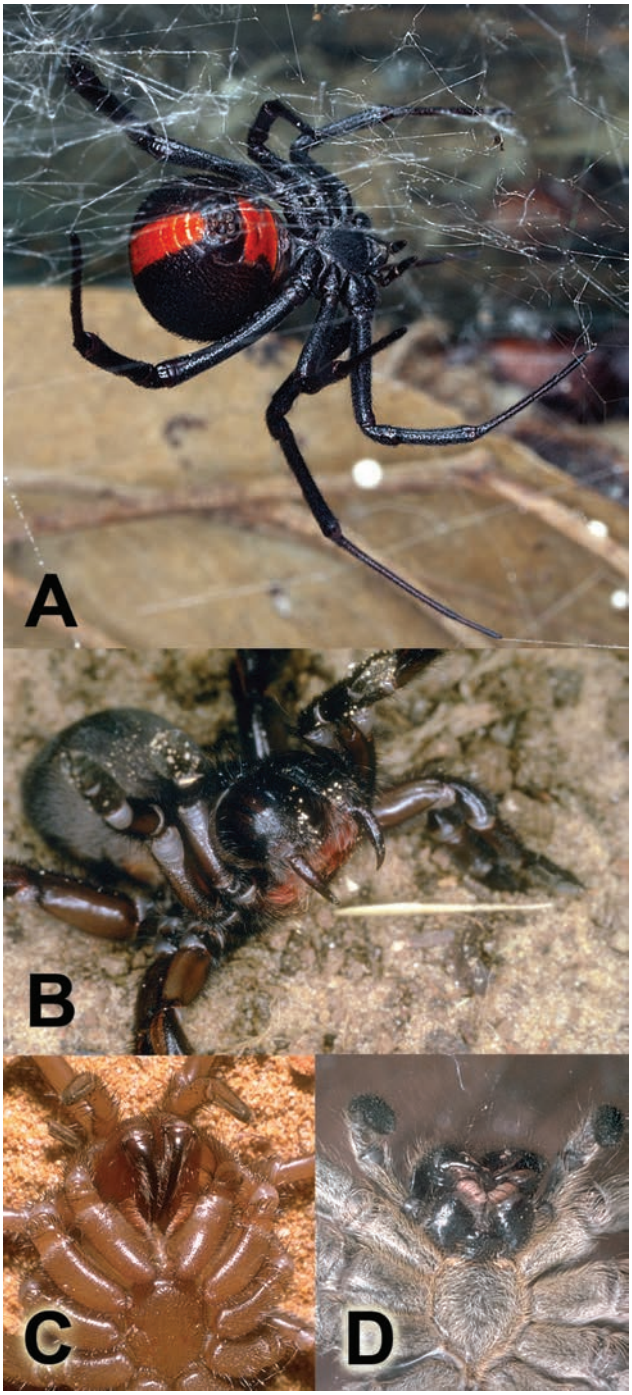


Figure 18-2. A medically important widow spider (*Latrodectus* spp) and comparison of the vertically deployed venom delivery apparatus of a mygalomorph spider and the horizontally deployed venom delivery apparatus of an araenomorph spider. (**A**) Red back widow spider (*Latrodectus hasselti*, *Theridiidae*). (**B**) Fangs of the mygalomorph, *Aganippe subtristis* (four-spotted trapdoor spider, female specimen, family Idiopidae). Note the vertical direction of the fang-bearing chelicerae traditionally termed paraxial, with the spider in an adopted defensive posture. (**C**) Fangs of the mygalomorph, *Aganippe subtristis*. The figure again illustrates the vertical direction of the fang-bearing chelicerae. (**D**) Fangs of the araenomorph, *Pediana* spp (a taxon of huntsman spider, family *Sparassidae*). The figure illustrates the horizontal deployment of the chelicerae-bearing fangs traditionally termed diaxial.
Photographs: Copyright © Julian White. In: White J. *A Clinician's Guide to Australian Venomous Bites and Stings*. Melbourne, Australia: Commonwealth Serum Laboratories; 2013: 300+ pp.



Figure 18-3. Brown recluse spider (*Loxosceles reclusa*). One of two genera (*Sicarius* and *Loxosceles*) belonging to the family *Sicaridae*, there are approximately 113 recognized taxa of *Loxosceles*. Several of these, including *L reclusa*, have inflicted medically significant bites that may occasionally cause a recurrent ulcer. In some parts of South America, several species may cause systemic envenoming (viscerocutaneous loxoscelism), an uncommon but potentially life threatening venom disease. There is antivenom available in several Latin American countries (eg, Brazil, Chile, Peru, Mexico), but treatment in the United States remains somewhat controversial with no quality evidence supporting the previous management (eg, surgical debridement, etc) of verified *L reclusa* bites. Meticulous wound management and possibly bariatric oxygen treatments are the most appropriate management methods. Recluse spider bites are among the most misdiagnosed presentations to emergency departments and outpatient/urgent care facilities, and diagnosis must be founded on verified identification of a spider or, in lieu of a specimen, with a well-supported history of a bite occurring within the range of recluse spiders (many suspected bites have occurred well outside any natural range or region in which the species has been accidentally introduced).
Photograph: Copyright © Julian White. In: White J. Venomous animals: clinical toxinology. *EXS.* 2010;100:233–291. In: Luch A (ed). *Molecular, Clinical and Environmental Toxicology*. Vol 2: *Clinical Toxicology*. Basel, Switzerland: Birkhäuser; 2010.

the family Ixodidae (hard-bodied ticks), particularly including members of the genera *Ixodes*, *Amblyomma*, *Dermacentor*, and the less common family *Argasidae*, notably the genus *Argas* (soft-bodied ticks, which so far have caused paralysis in animals only, not humans).

Insects

Insect sting envenoming causing toxin-induced disease of medical significance is uncommon, but allergic reactions to hymenopteran insect stings (eg, anaphylaxis from ants, bees, wasps, and hornets) are common and sometimes fatal. Retrospective studies have suggested that typically 40 to 100 deaths occur each year from hymenopteran sting anaphylaxis in the United States.[18] This amount is a significantly higher annual patient fatality rate than that of snakebite envenoming in the United States (typically between 5 and 8 patients).

Some bee venoms (eg, bumblebee, *Megabombus pennsylvanicus*; honey bee, *Apis mellifera*; Figure 18-5) contain mast cell degranulating peptide, a 22-amino acid cationic peptide that can directly trigger release of proanaphylactic mediators without prior sensitization.

One unusual example of a medically important insect venom is the Lepidopteran larvae (caterpillar) of the giant silkworm moth, *Lonomia obliqua* (family *Saturniidae*; Figure 18-6), whose spines contain several direct and indirect prothrombin activators, as well as several other toxins.[19] The sting of this caterpillar can cause a hemorrhagic diathesis, and fatalities have been documented.[34] Other Lepidopteran larvae have been implicated in human disease, at least some of which may involve local envenoming. Other terrestrial venomous animals cause few cases of human disease.

Marine Envenoming: Sea Snakes, Cnidarians, and Venomous Fish

Marine envenoming, such as jellyfish stings, are common, but few are medically significant. Significant types of marine envenoming include box jellyfish (eg, *Chironex fleckeri*, family *Chirodropidae*) stings (sometimes lethal); Irukandji jellyfish stings (resulting in a syndrome caused by several taxa of cnidarians, rarely lethal; see below under Excitatory Neurotoxins); and blue bottle (*Physalia* spp, family *Physaliidae*) stings (nonlethal envenoming, but occasional cases of potentially lethal allergic reactions). Sea snake (family Elapidae) bites can cause lethal envenoming, but are increasingly uncommon because of changes in fishing methods (eg, decreased manual removal of snakes from purse nets). Painful stings from venomous fish from several different families (eg, *Scorpaenidae*, *Trachinidae*, and *Tetrarogidae*), including many popular food and aquarium fishes as well as marine and fresh or brackish water stingrays, are common but generally unlikely to be lethal.

Some 200 species of stingrays, which belong to seven of nine families, can deliver venomous stings, or more accurately termed, penetrative envenoming. The most medically important stingrays belong to the following families:



Figure 18-4. Sydney funnel-web spider (*Atrax robusta*). Approximately 13 fatalities have resulted from *A robusta* envenoming that clinically presents as a catecholamine storm produced by potent neuroexcitatory venom toxins.
Data source: White J. *A Clinician's Guide to Australian Venomous Bites and Stings*. Melbourne, Australia: Commonwealth Serum Laboratories; 2013.
Photograph: Copyright © Julian White. In: White J. *A Clinician's Guide to Australian Venomous Bites and Stings*. Melbourne, Australia: Commonwealth Serum Laboratories; 2013: 300+ pp.

- *Urolophidae* (stingarees),
- *Dasyatidae* (whiptail stingrays),
- *Hexatrygonidae* (sixgill stingrays),
- *Potamotrygonidae* (river stingrays), and
- *Plesiobatidae* (giant stingrays).

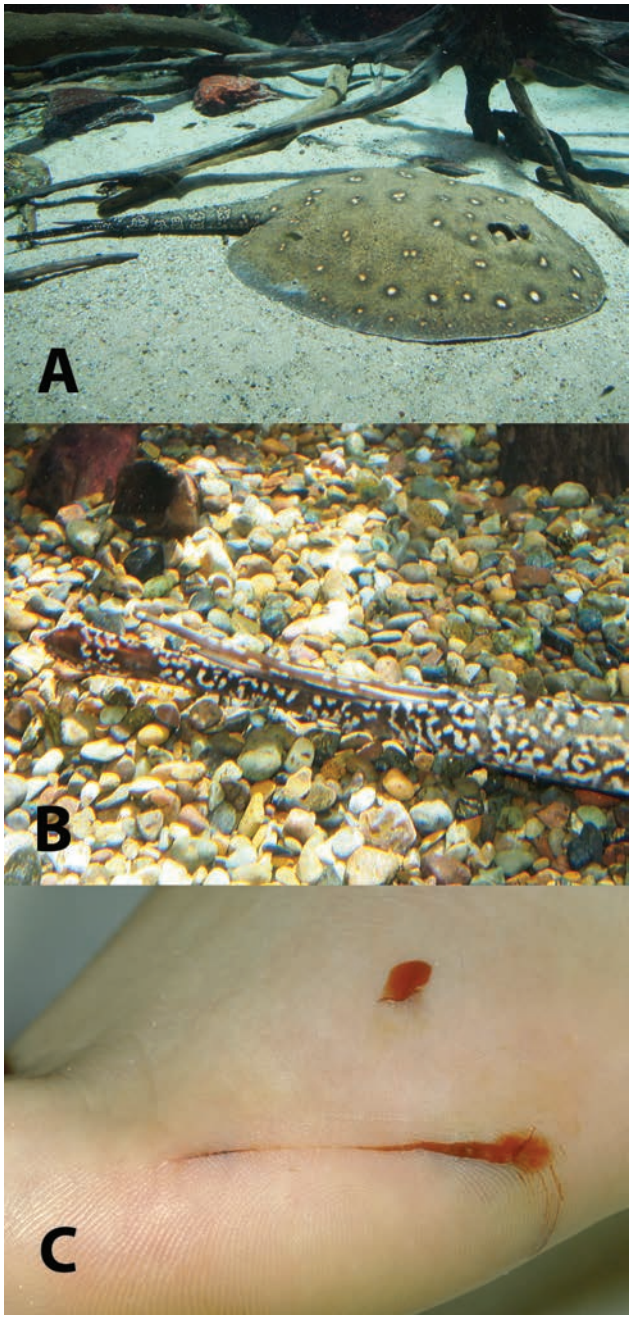
Stingrays, which are cartilaginous relatives of sharks (all in the class Elasmobranchii) and do not possess venom glands, deliver their stings using a serrated spine that is covered with mucosal secretions and venom-secreting cells (Figure 18-7A, B). The cells release the venom into the wound produced by the spine penetration (penetrative envenoming). The venom contains cytotoxic and vasculotoxic (including probably cardiotoxic) properties, and secondary infection from the wounds is common. Laceration of the lower extremities (especially the foot and ankle) is common (Figure 18-7C) with later clinical evolution of edema, cellulitis, and occasionally necrosis. Rare deep penetrative envenoming from giant species such as the Australian smooth stingray (*Dasyatis brevicauda*, family *Dasyatidae*) can be fatal if the thoracic cavity is pierced. In these uncommon cases, fatal effects usually result from physical trauma rather than envenoming, although, as mentioned previously, some experimental data have demonstrated cardiotoxicity of some stingray venoms (see Mebs, 2002 and Meier and White, 1995).[34,35] The well-known television personality Steve Irwin succumbed rapidly to the intracardiac



Figure 18-5. Honeybee (*Apis mellifera*). Stings from hymenopterans (especially bees and wasps) can cause life-threatening anaphylaxis in susceptible individuals. There are significantly more annual fatalities in the United States from hymenopteran sting-induced anaphylaxis than from snakebite envenoming.
Data sources: (1) Weinstein SA, Dart RC, Staples A, White J. Envenomations: an overview of clinical toxinology for the primary care physician. *Am Fam Physician*. 2009;80:793–802. (2) Weinstein SA, Warrell DA, White J, Keyler DE. *"Venomous" Bites from Non-venomous Snakes. A Critical Analysis of Risk and Management of "Colubrid" Snake Bites*. 1st ed. New York, NY: Elsevier; 2011.
Photograph: Copyright © Julian White. In: White J. *A Clinician's Guide to Australian Venomous Bites and Stings*. Melbourne, Australia: Commonwealth Serum Laboratories; 2013: 300+ pp.



Figure 18-6. Larvae of the giant silkworm moth (*Lonomia obliqua*). The larvae of this moth can inflict a life-threatening envenoming that features coagulopathy.
Photograph: Centro de Informações Toxicológicas de Santa Catarina, Brazil. Wikipedia Commons, public domain. http://www.cit.sc.gov.br.



penetration from a giant Australian stingray, and his death most likely resulted from cardiac tamponade, not envenoming.

Poisoning by Animals, Plants, and Mushrooms

Poisonings from ingestion of poisonous animals, especially marine animals, are common. These animals include the fugu puffer fish (eg, *Takifugu* spp, family *Tetraodontidae*); most species of fish that belong to the order Tetraodontiformes, which contain tetrodotoxin; ciguatoxic fish (numerous species, including many colorful diverse reef species and apex predators such as the great barracuda, *Sphyraena barracuda*); and several types of shellfish. In some Pacific island populations, ciguatera poisoning affects up to 1 in 5 people each year, and from 1973 to 2008 an estimated 500,000 people were affected by ciguatera poisoning.[20] Some types of marine poisoning carry a significant fatality rate, particularly the ingestion of sushi made with fugu (especially when including visceral organ meats), which equates with tetrodotoxin poisoning (see Mebs, 2002 and Meier and White, 1995).[34,35] Some types of shellfish poisoning also have a substantial risk of death (see Mebs, 2002).[34]

Poisoning from ingestion of poisonous plants and mushrooms, which is similarly common, is particularly frequent or important in some regions, notably parts of the tropics (poisonous plants) and in Europe and parts of North America (poisonous mushrooms). It can occur as a consequence of accidental circumstances (misidentification of the plant/mushroom) or as a deliberate act (eg, use of oleander [eg, *Thevetia peruviana*, family *Apocynaceae*] ingestion in suicides in the Indian subcontinent).[21,22] Some types of plant or mushroom poisoning carry a relatively high fatality rate, especially in delayed or late presentations; however, this may reflect regional trends because in some Western countries (eg, the United States), the case fatality rate for mushroom poisoning remains low.[23]

Figure 18-7. South American freshwater or river stingray (*Potamotrygon motoro*), venom apparatus and stingray penetrative envenoming. (**A**) Ocellate or peacock-eye river stingray (*Potamotrygon motoro*). This increasingly rare species is popular among home aquarists. It is capable of inflicting a sting that can cause moderate to severe local effects, including severe pain, and systemic effects including shock. (**B**) Stingray tail spine. Stingrays do not possess a venom gland; rather, their serrated tail spines have venom-containing cells that release their contents when physically disrupted. The spine also is coated with a mucous layer that may be colonized with several taxa of marine microorganisms and can predispose to serious local infection in an envenomed victim. (**C**) Stingray spine (species unidentified)-inflicted wound on foot. Wounds may result from directly stepping on a ray covered with sand, or from a glancing, slash-like wound. These wounds often require medical imaging in order to determine if any spine fragments remain imbedded in the wound, as well as meticulous wound care and prophylactic antibiotics.
Photographs: Copyright © Julian White. In: White J. *A Clinician's Guide to Australian Venomous Bites and Stings*. Melbourne, Australia: Commonwealth Serum Laboratories; 2013: 300+ pp.

Relevance to Biological Warfare

Venomous animals generally evolved to target prey or predators on an individual basis—not en masse—so they are not readily adapted to act as ideal weapons in human warfare. However, either using the native toxins or modifying those toxins to enhance a particular action and developing an artificial weaponization and delivery strategy is possible, but presents logistical challenges that would likely outweigh practicality in most cases.

Venoms contain some highly potent agents that can kill humans in small doses. In most cases the lethal outcome will not be instantaneous, but likely prolonged over several hours. Venoms are not ideal overall for biological warfare because of these delivery problems as well as the absorption/direct administration required in most examples to optimize their actions. Even for potent neurotoxins, such as paralytic or neuroexcitatory toxins, other equally or more potent chemicals are available that allow mass delivery. Also, most venom-derived toxins are susceptible to thermolability and varying degrees of denaturation through other environmental influences that can affect their potency. Some of them also exhibit nonmammalian prey target specificity, for example, some antagonize the acetylcholine receptors of lizards and birds. Thermostable toxins are found in a few venoms such as those of the venomous helodermatid lizards (*Heloderma* spp; see below) and the unusual peptide neurotoxins from temple or Wagler's pit viper (*Tropidolaemus wagleri*) venom.[24,25] Some of the specific factors influencing the use of biological toxins in biowarfare and preparedness against such a threat have been discussed by Osterbauer and Dobbs.[26]

Research into specific toxins, including their molecular modification, and possible xenogenic incorporation of toxin-encoding genes into potentially infectious microorganisms may allow substances to be developed with biological warfare potential. Several countries have explored the potential uses for such recombinant products. The ethics and realities of such research are beyond the scope of this chapter.

Similarly, toxins from poisonous animals, plants, and fungi may—in general—be unattractive as biological warfare agents, although ricin (from the castor bean plant, *Ricinus communis* [family *Euphorbiaceae*]) and the aflatoxins (from the molds, *Aspergillus* spp, family *Trichocomaceae*) are exceptions. Specifically excluded from this discussion is the casual/accidental interaction between combatants and venomous fauna on the battlefield or in otherwise deployed locations.

However, the risk posed by accidental envenoming, especially snakebite and scorpion sting, should not be overlooked in developing risk-mitigation strategies for any potential combat zone. Some authors have described the impact that venomous animals may have on field troops. Maretic[27] reported several accounts of mass envenoming of large numbers of troops by widow spiders, *Latrodectus* spp. These troops include the troops of Ludwig in Calabria in 866 CE who were "decimated" by spiders. Also, during the eve of the Battle of Loncomilla that occurred during the Chilean Revolution on the 8th of December 1851, soldiers bitten by *Latrodectus* spp were chloroformed so as not to "betray with their screams" the position of the army.[27]

However, the general concerns about risks posed by venomous animals to modern troops deployed in locales with several medically important venomous species have appeared to be disproportionate to the small number that are seriously or fatally envenomed. For example, Ellis[28] reported only three recorded snakebite-related deaths among British troops during World War II, and Minton and Minton[29] reported only one well-documented fatal snakebite inflicted on an American soldier during the Vietnam War. It is likely that such cases were underreported and the actual numbers of those less seriously envenomed are unknown. Although few figures account for envenomings among coalition troops in Operations Desert Shield or Desert Storm, the Persian Gulf War, or Operation Enduring Freedom in Afghanistan, there are a handful of documented cases. Two enlisted American military service personnel were among 17 snakebite victims treated at three US medical facilities in Afghanistan. Most of the patients in this series were local Afghans, and the identity of the envenoming snake species was unknown in 11 of 17 cases (65%).[30] There were no fatalities, and 10 of 17 patients (58%) received antivenom.[30]

However, in some circumstances, natural disasters may share some features with the effects of warfare on civilian populations. Envenoming can become a significant risk in certain disasters. The effects of massive flooding in Bangladesh are well studied because it is a frequent disaster event. Although drowning is the single most common reason for fatalities in floods by a substantial margin, snakebite is the second most common reason for fatalities and causes as many or more deaths than all other causes (except drowning) combined.[31] The diagnosis, first aid, medical treatment, and prevention of such accidental envenoming are major subjects beyond the scope of this chapter, although some basic recommendations are presented.

MAJOR TOXIN CLASSES AND THEIR CLINICAL EFFECTS

There are many ways of classifying toxins, including by taxonomic origin, chemical structure, molecular targets, and biological activity. For this chapter, a pathophysiologically based scheme is most relevant in considering the primary actions of venom toxins and possible clinical presentations that may occur as a result of their action.

Paralytic Neurotoxins

For venomous animals, paralysis is a biologically useful state to induce in either prey or predator. For some arthropods, paralyzing prey allows them to both overcome larger prey and provide a food store for leisurely later feeding, or for their offspring to feed on during their larval stage. For cone snails (*Conus* spp, family *Conidae*; Figure 18-8), the use of paralytic neurotoxic peptides delivered by ejection of a harpoon-like modified radula tooth allows this slow moving predator to capture and ingest fast moving prey (fish). For most cephalopods (eg, octopuses) and some squamate reptiles (eg, venomous snakes), the use of neurotoxins delivered respectively by beak or canaliculated fang (containing an internal lumen)/externally grooved modified maxillary teeth (Figure 18-9) allows safe subjugation and consumption of prey (eg, respectively, crabs, mammals) that otherwise might injure the octopus/snake.



Figure 18-8. Textile cone snail (*Conus textilis*). These gastropods are highly coveted by amateur conchologists for their beautiful shells. There are rare human deaths from stings delivered by these snails when they are handled. A modified tooth, the radula that closely resembles a miniature harpoon, delivers the venom into prey or an unfortunate human victim. This adaptation allows this slow-moving snail to capture fast moving fish. Various *Conus* species (>650 currently recognized) often favor specific prey such as fish or other gastropods (including other *Conus* spp).
Photograph: By Jan Delsing. Wikipedia Commons, public domain. https://commons.wikimedia.org/wiki/File:Conus_textile_010.jpg.

For poisonous animals, the effects of neurotoxins may either permanently deter potential predators or allow escape by the poisonous animal. The theoretical evolutionary value for poisonous plants and mushrooms is less clear, although it can be speculated that excessive foraging of these species may have occurred and endangered their survival. Perhaps these plants produce noxious and toxic substances to discourage their consumption. Also, these toxins may serve (or have served) other functions important to the physiological functions of the plant that are unrecognized. The costs of evolving such a biosynthetic capability may be a factor contributing to the less common occurrence of such toxins in these taxa.

The mechanism that causes paralysis is variable, but in most cases it involves direct toxin activity in the peripheral nervous system, rather than a central effect. The molecular variability in these toxins is considerable, but within particular venomous animal groups tends to be more homogenous and conserved. The best described examples are snake venom neurotoxins, many of which have been duplicated among different ophidian clades (Table 18-2).[32–35]

There are essentially four principal snake paralytic neurotoxin types of clinical significance (see Table 18-2). Most are polypeptides, some have multimeric structures that include one or more basic phospholipases A2 (PLA2) subunits, and a few unusual neurotoxins are small peptides. Within each type, some molecular variability exists, particularly among presynaptic neurotoxins, which are generally the most potent though slightly slower acting paralytic toxins. Postsynaptic neurotoxins generally fall within one of two classes that are commonly termed short and long chain, but these are now collectively classed as "three-finger-fold" toxins in reference to the three β-stranded loops extending from their central core that contain all four conserved disulfide bonds. These toxins most commonly have molecular masses ranging from 6 to 8 kDa (see Table 18-2). The other well-characterized toxins such as the fasciculins (a group of anticholinesterases that have the three-finger fold conformation) and dendrotoxins (neuronal voltage-gated potassium channel inhibitors; see Table 18-2) from mamba venoms act differently from each other, but are synergistic. All act at the neuromuscular junction (Figure 18-10).[32–35] The cited references offer more detailed reviews of these essential toxin classes.

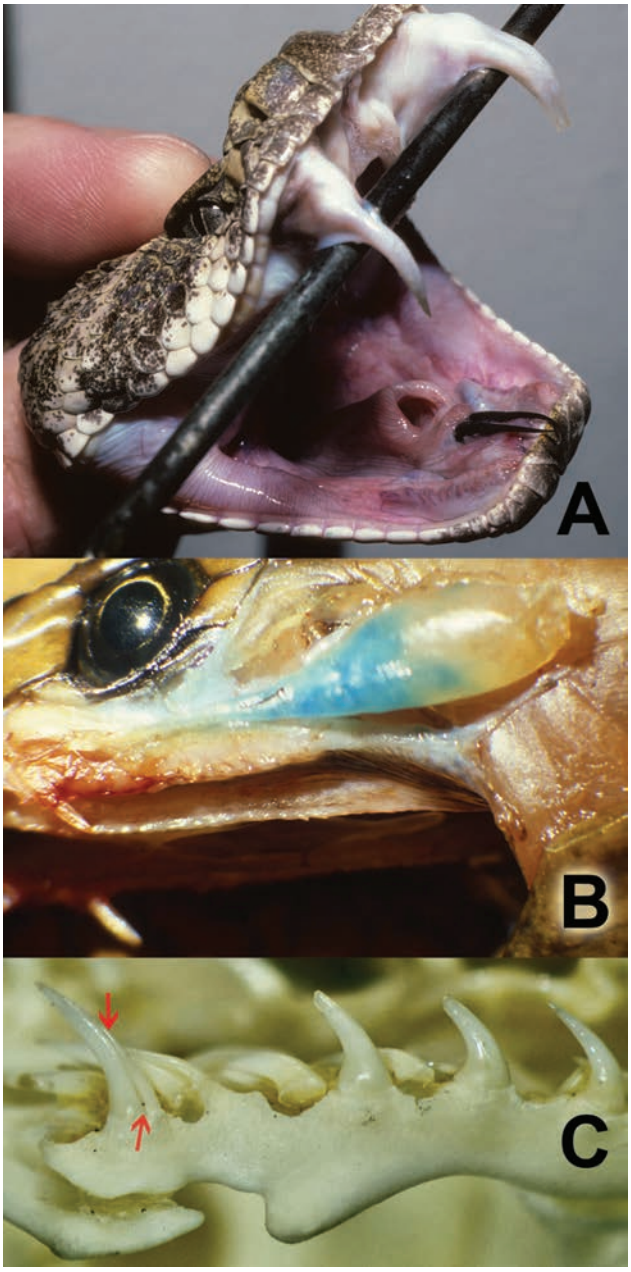


Figure 18-9. Representative venom delivery apparatuses found among some snakes. (**A**) Distensible fangs of a representative viperid, Western diamondback rattlesnake (*Crotalus atrox*). This dentitional arrangement has often been termed solenoglyphous. Elapids, viperids, and front-fanged lamprophiid snakes are collectively termed front-fanged colubroids. (**B**) Fixed fangs of a representative elapid, Eastern brown snake (*Pseudonaja textilis*). This dentitional arrangement has often been termed proteroglyphous. (**C**) The posterior grooved maxillary teeth of a non–front-fanged colubroid, the Mangrove or ringed cat eye snake (*Boiga dendrophila*). The maxillary is placed upside down to better illustrate the characteristics of the teeth. The deep external grooves conduct the venom from the low-pressure glands associated with the delivery apparatus. This dentitional arrangement that can include mid- or notably posterior maxillary teeth that may be enlarged and may also be grooved has been termed opisthoglyphous, or rear fanged with aglyphous referring to those non–front-fanged colubroids that have mid or posterior teeth that lack grooves and in some instances are also associated with a low pressure gland. These terms are not precisely accurate because the modified dentition may occur midway in the maxillary.
Photographs: (**A**) Copyright © Julian White. In: Brent J, Wallace KL, Burkhart KK, et al (eds). *Critical Care Toxicology: Diagnosis and Management of the Critically Poisoned Patient*. 1st ed. Philadelphia, PA: Mosby; 2005. (**B**) Copyright © Julian White. In: Covacebich J, Davie P, Pearn J (eds). *Toxic Plants and Animals: A Guide for Australia*. Brisbane, Australia: The Queensland Museum; 1987. (**C**) Copyright © Scott A Weinstein. In: Weinstein SA, Warrell DA, White J, Keyler DK. *"Venomous" Bites from Non-Venomous Snakes: A Critical Analysis of Risk and Management of "Colubrid" Snake Bites*. New York: Elsevier; 2011: 141.

Other neurotoxins, such as tetrodotoxin and saxitoxin, are nonproteinaceous and low molecular mass (often between 300 and 1,000 Da) toxins (the former is a guanidinium class toxin, the latter a polyether), and thus have different structures as well as mechanisms of action. These are mostly ion channel toxins that are very potent with notably low minimal concentrations required to affect biological activity.[36,37] Small amounts ingested (eg, through eating fugu fish [tetrodotoxin] or contaminated shellfish [eg, saxitoxins, yessotoxin]) can result in rapid complete paralysis, but the paralysis is usually of shorter duration, in comparison with snake venom neurotoxins.[33,38–40]

The biothreat potential of tetrodotoxin has been long recognized. It is included on the US Department of Health and Human Services' regulated select agent list and considered an agent "determined to have the potential to pose a severe threat to both human and animal health."[41,42]

Clinically, these paralytic neurotoxins cause grossly similar presentations, with progressive development of flaccid paralysis. In most cases a descending paralysis affecting cranial nerves occurs first (however, envenoming by paralytic tick species causes an ascending paralysis commencing with ataxia).[40,43–45] In a classic presentation after snakebite, the patient develops bilateral ptosis after one to several hours, which may progress to complete ophthalmoplegia and fixed dilated pupils if untreated, although even timely treatment does not always prevent this progression. Dysarthria, dysphagia, drooling, and loss of upper airway protection may occur, followed by limb muscle weakness, loss of deep tendon reflexes, diaphragmatic paralysis, and complete respiratory paralysis. Without intubation

TABLE 18-2

SOME PRINCIPAL PARALYTIC NEUROTOXIN TYPES

Toxin Class	Structure	Site of Action	Mode of Action	Source	Examples
Presynaptic neurotoxins	PLA2-based, mono- or multimeric	NMJ	Bind to surface membrane of terminal axon, modify SNAP proteins, enter the axon via synaptosomes, and then damage mitochondria and other cell structures, thus disrupting synaptosome production, thereby causing complete paralysis	Many elapid snakes (Australian elapids, coral snakes, kraits); a few vipers (South American rattlesnakes, a few North American rattlesnakes, some "old world" viperids, such as Russell's vipers)	Notexin (Australian tiger snake, *Notechis scutatus*); Mojave toxin (Mojave rattlesnake, *Crotalus scutulatus*); alpha-bungarotoxin (widespread in studied venoms from kraits, *Bungarus* spp)
Postsynaptic neurotoxins	Polypeptide, with variable number of disulfide bonds; variably termed, long-chain, short-chain, or three-finger-fold neurotoxins	NMJ	Bind to acetylcholine receptor on muscle end plate and cause reversible or irreversible block preventing activation of receptor	Many elapid snakes (Australian elapids, sea snakes, coral snakes, cobras, kraits) and some non–front-fanged colubroid snakes (NFFCs); studied NFFC toxins have so far been largely prey-specific	Long-chain or short-chain neurotoxins from banded water cobra (*Naja annulata*), black-necked spitting cobra (*N nigricollis*) and many others
Dendrotoxins	Polypeptides that are structurally homologous with Kunitz-type proteinase inhibitors	NMJ	Cause massive release of acetylcholine from terminal axons through activation of potassium channels, flooding the junctional space, and receptors	African mambas (*Dendroaspis* spp)	Eastern green mamba (*D angusticeps*)
Fasciculins	Three-finger-fold polypeptides	NMJ	Prevent regulated removal of acetylcholine from the junctional space, thereby overstimulating and inactivating receptors causing muscle fasciculation	African mambas (*Dendroaspis* spp)	Black mamba (*D polylepis*)
Tetrodotoxins	Steroidal alkaloid (Guanidinium class)	Na^+ channels in excitable nerve and muscle cells	Causes blockade of the voltage-gated sodium channels (NaV) in nerve and muscle cell membranes by binding to site 1 of the NaV α-subunit, thus blocking ion conduction and preventing the cells from activation, as well as inhibiting release of neurotransmitter	Blue-ringed octopus, puffer (fugu) fish, selected newts, toads, flatworms, and a diverse series of other animals; the toxin with the broadest phylogenetic distribution; probably produced in some species by symbiotic bacteria (eg, present in the beak-associated venom/salivary glands of blue-ringed octopuses and relatives)	Greater blue-ringed octopus (*Hapalochlaena lunulata*)

(**Table 18-2** *continues*)

Table 18-2 continued

Batrachotoxins, homobatrachotoxins	Steroidal alkaloid with oxazepine ring	Axolemma	Binds to site 2 of the NaV α-subunit, thereby increasing the permeability of the voltage-dependent sodium channel by prolonging the open state; this causes persistent activation and shifted voltage dependence; the toxin has approximately 10- to 12-fold greater experimental lethal potency than tetrodotoxin	Poison dart frogs (*Dendrobatidae*), pitohui birds (PNG); the dendrobatid frogs obtain these toxins from insect food sources (eg, coleopteran, hymenoptera, and others), and the toxin becomes absent in specimens maintained on nonindigenous insects in captivity	Yellow poison-dart frog (*Phyllobates terribilis*)
Saxitoxins, gonyautoxins; others	Purine derivatives (polyethers: the molecular structures classify into groups based on potency [most potent to least]; carbamates, decarbameyl, *N*-sulfacarbamyl, hydroxybenzoate)	Excitable cell membranes	Bind adjacent to the sodium channel, blocking the channel and preventing action potentials; like tetrodotoxin, saxitoxin exerts its activity by binding to site 1 of the NaV α-subunit	Selected shellfish (paralytic shellfish poisoning); toxins are produced by a wide variety of dinoflagellates and bioconcentrated in filter-feeding shellfish; some toxins may be produced in cyanobacteria and algal spp	Saxitoxin is produced by an indeterminate number of marine picoplankton, such as the dinoflagellates, *Gymnodinium*, *Alexandrium*, *Pyrodinium* and others; gonyautoxin is produced by some of the aforementioned species and others; these and other toxins have also been detected in cyanobacterial blooms in fresh water
Holocyclotoxins (HT-1)	Probably several isotoxins (HT-1, HT-2, HT-3, and possibly others); HT-1 is a basic polypeptide with a calculated molecular mass of 5.9 kDa	NMJ	Similar to snake venom presynaptic neurotoxins	Paralysis ticks in Australia, North America, and Southern Africa	Australian paralysis tick (*Ixodes holocyclus*)
Conopeptides	Broad array of peptides		A variety of mechanisms, depending on toxin; as an example, the μ-conotoxins bind to site 1 of the NaV α-subunit	Selected *Conus* spp cone snails; an indeterminate number of the >650 species produce these toxins; of those tested, many produce toxins that are prey-specific for either fish or invertebrates, including other molluscs; only a handful produce toxins that are medically significant in humans	Geographer or geography cone (*Conus geographicus*)

NMJ: neuromuscular junction; PLA2: phospholipase A2; PNG: Papua New Guinea; SNAP: synaptosomal-associated protein

Data sources: (1) Weinstein SA, Warrell DA, White J, Keyler DE. '*Venomous' Bites from Non-venomous Snakes. A Critical Analysis of Risk and Management Management of 'Colubrid' Snake Bites*. 1st ed. New York, NY: Elsevier; 2011. (2) Mebs D. *Venomous and Poisonous Animals: A Handbook for Biologists, Toxicologists and Toxinologists, Physicians and Pharmacists*. Boca Raton, FL: CRC Press; 2002: 360. (3) Meier J, White J. (eds). *Handbook of Clinical Toxicology of Animal Venoms and Poisons*. Boca Raton, FL: CRC Press; 1995. (4) Synthesized professional presentation materials (eg, lectures) of the authors.

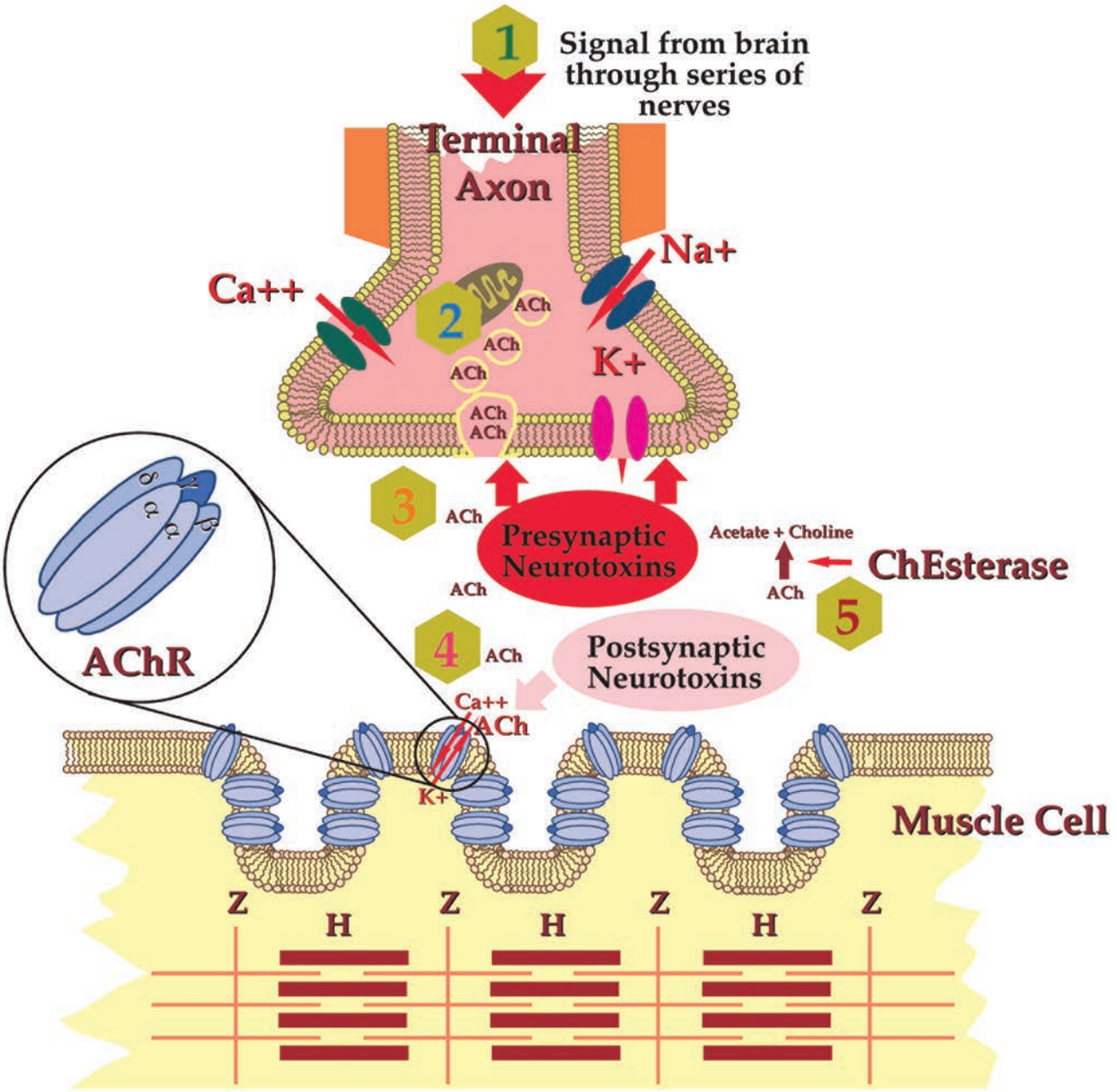


Figure 18-10. Sites of action of principal types of snake venom neurotoxins. Simplified overview of the general actions of phospholipase A2-multimeric presynaptic neurotoxins and postsynaptic neurotoxins. The *numbered steps* included in the figure indicate the following: (*1*) shows the initiation of translation of the electrical impulse into a biochemical release; (*2*) synaptic vesicles fuse with the axonal membrane thereby leading to exocytic discharge of acetylcholine (ACh); (*3*) discharged ACh enters the synaptic cleft; (*4*) the discharged ACh binds to motor end-plate receptors of the myocyte membrane leading to stimulation of contraction; and (*5*) the ACh at the motor end plate is then hydrolyzed by acetylcholinesterases, thus terminating the contractile stimulus. Note the site of respective site of actions of the presynaptic and postsynaptic neurotoxins. The investigation of presynaptic neurotoxin pharmacology has largely focused on the actions of several potent snake venom neurotoxins isolated from Australian or Asian elapids, as well as a few viperids (several species of rattlesnakes and viperine viperids). Some of the well-studied toxins—such as taipoxin, β-bungarotoxin, and crotoxin—ultimately inhibit ACh release at the neuromuscular junction, and in nerve-muscle preparations accomplish this in three phases: (1) an initial transient decrease/inhibition of evoked transmitter release that is promoted by Ca^{2+} (this phase has been absent in studies of some toxins, eg, notexin), (2) a facilitated transmitter release phase, and (3) the final phase that features a progressive fall in evoked

(**Figure 18-10** *continues*)

Figure 18-10 continued

release resulting in transmission failure and paralysis. Miniature end-plate potential frequency is similarly affected, although spontaneous release tends to occur at a low frequency after the failure of evoked neuromuscular transmission. However, spontaneous release amplitude does not change significantly, suggesting that synaptic vesicles do not fuse inside the axonal terminal and that the ACh-synaptosomal packaging mechanism is not impaired by the action of the studied species of these toxins. The phospholipase A2 subunit(s) in some of these toxins (eg, Mojave toxin) also have a degenerative effect on the motor end plate. There is still controversy about the specific enzymatic influence of phospholipase A2 subunits on the neurotoxicity and the initial phospholipid hydrolysis role in initiating the three-phase mechanism that ultimately results in paralysis, but the lysophospholipids produced by hydrolysis do alter the active zones of neuroexocytosis thereby making them less prone to membrane fusion with synaptic vesicles. Unlike the actions of several botulinum toxins (eg, botulinum toxins A and E) from *Clostridium botulinum* and tetanus toxin from *C tetani* that function as endopeptidases by cleaving integral proteins (eg, SNAP-25; other botulinum toxins cleave VAMP or SNAP-25 and syntaxin) of the presynaptic membrane, snake venom phospholipase A2-containing neurotoxins do not directly hydrolyze these SNARE proteins. Although other presynaptically acting neurotoxins, such as α-latrotoxin from the widow spider (*Latrodectus* spp) venoms function with a mechanism different from that of snake venom phospholipase A2 toxins, these different toxins still alter the axolemmal permeability and cause Ca^{2+} overload within the terminals and subsequent neuronal degeneration, a process that likely causes activation of the calcium-activated proteolytic activities of calpains. The increased Ca^{2+} permeability induced by α-latrotoxin occurs via toxin binding to its receptor(s) (eg, latrophilin) subsequently forming pores in the presynaptic membrane. The terminal is thus essentially flooded with Ca^{2+} and vesicle fusion is over-stimulated resulting in a massive release of neurotransmitter. Most snake venom postsynaptic neurotoxins bind to subunit interfaces in either muscle type (α-1) or neuronal type (α-7) nicotinic Ach receptors (AChR), thereby antagonizing neurotransmitter binding with resultant paralysis. The length of their primary sequence, long-chain and short-chain, has been commonly used to classify these toxins but recently all of these toxins have been renamed as three-finger-fold neurotoxins. The concave aspect of the three-dimensional structure of these toxins contains several amino acids that function as active sites for binding to respective AChR subunits. These toxins are widespread among elapid venoms and some are found in venoms of other snakes such as some colubrids and lamprophiids, as well as a few viperids (eg, *Daboia russelii*). Snake venom neurotoxicity should not be viewed as a result of the isolated action of discrete toxins such as those outlined above. For example, fasciculins and dendrotoxins from mamba (*Dendroaspis* spp) venoms can facilitate the subjugation of prey animals, as well as compound the clinical manifestations in envenomed patients. Fasciculins are reversible, selective acetylcholinesterase antagonists that cause an accumulation of acetylcholine at the neuromuscular junction thereby causing marked and prolonged fasciculations. Pita et al considered the potential use of fasciculins, as well as other anticholinesterase toxins, as biological warfare agents. Originally isolated from venom of the Eastern green mamba (*D angusticeps*), dendrotoxin is a 7-kDa polypeptide homologue of Kunitz-type serine protease inhibitors. However, unlike mammalian Kunitz-type inhibitors, the dendrotoxins are potent, selective blockers of voltage-dependent potassium channels. These toxins induce repetitive and sustained terminal neuronal firing, and have increasingly been used as molecular tools in ion channel neuropharmacology.

ChEsterase: cholinesterase; SNARE: soluble *N*-ethylmaleimide-sensitive factor activating protein receptor; VAMP: vesicle-associated membrane protein

Data sources: (1) Pungerčar J, Križaj I. Understanding the molecular mechanism underlying the presynaptic toxicity of secreted phospholipases A2. *Toxicon*. 2007;50:871–892. (2) Rossetto O, Montecucco C. Presynaptic neurotoxins with enzymatic activities. *Handb Exp Pharmacol*. 2008;184:129–170. (3) Duregotti E, Tedesco E, Montecucco C, Rigoni M. Calpains participate in nerve terminal degeneration induced by spider and snake presynaptic neurotoxins. *Toxicon*. 2013;64:20–28. (4) Davletov B, Ferrari E, Ushkaryov Y. Presynaptic neurotoxins: an expanding array of natural and modified molecules. *Cell Calcium*. 2012;52:234–240. (5) Teixeira-Clerc F, Ménez A, Kessler P. How do short neurotoxins bind to a muscular-type nicotinic acetylcholine receptor? *J Biol Chem*. 2002;277:25741–25747. (6) Harvey AL, Robertson B. Dendrotoxins: structure–activity relationships and effects on potassium ion channels. *Curr Med Chem*. 2004;11:3065–3072. (7) Pita R, Anadón A, Martínez-Larrañaga MR. Neurotoxins with anticholinesterase activity and their possible use as warfare agents. *Med Clin (Barcelona)*. 2003;121:511–517.

Illustration: Copyright © Julian White. In: White J. Venomous animals: clinical toxinology. *EXS*. 2010;100:233–291. In: Luch A (ed). *Molecular, Clinical and Environmental Toxicology*. Vol 2: *Clinical Toxicology*. Basel, Switzerland: Birkhäuser; 2010.

and mechanical ventilation, this condition is fatal. It may take many hours to reach this final, potentially fatal stage. With external respiratory support, most affected patients can be expected to survive.

If the paralysis is caused by presynaptic snake venom neurotoxins, then complete paralysis may extend to days and weeks (but rarely months) until the damaged terminal axons at the neuromuscular junction regenerate. Antivenom cannot reverse this process and is only effective if given early enough to neutralize the neurotoxins before they bind and enter the terminal axon.

If the paralysis is caused by only postsynaptic snake neurotoxins, then the blockade may sometimes be reversed by giving antivenom, or it can be moderated by increasing the supply of neurotransmitter

(acetylcholine) from the terminal axons. This can sometimes be accomplished by administering anticholinesterases (usually intravenous neostigmine) to partially overcome the receptor blockade.

For poisoning from the nonprotein neurotoxins such as tetrodotoxin, the patient is most often wholly reliant on mechanical ventilation that constitutes the only treatment during major paralysis. However, complete paralysis and the concomitant artificial ventilation may often last only a few hours. These toxins act rapidly and can cause complete respiratory paralysis within 20 to 60 minutes of toxin exposure; thus the onset of symptoms and signs is precipitous.

Excitatory Neurotoxins

Excitatory neurotoxins cause nonparalytic stimulation of the nervous system and may exert their effect on many or all parts of the peripheral nervous system, including the autonomic nervous system. These are the classic arthropod toxins found in selected scorpion and spider venoms. They also occur in some other venoms, such as Irukandji jellyfish venoms.

Most are potent and highly selective ion channel toxins, variously affecting sodium, potassium, and calcium channels and either activating or blocking these channels. For example, the δ-atracotoxins from *Atrax robusta* (Sydney funnel-web spider) venom interact with a specific voltage sensor transmembrane segment (S4) of α-subunit domain IV. The interaction of the δ-atracotoxins with S4 prevents the normal outward ionic movement, and associated conformational changes that are required for channel inactivation. This results in prolonged action potentials at autonomic or somatic synapses, which induces massive transmitter release.[46] Many excitatory toxins have become vital tools in unlocking the secrets of nerve signaling at the molecular level because of their specific mechanisms, and they are the subject of ongoing intensive research. Most are low molecular weight peptides (eg, the aforementioned δ-atracotoxins consist of 42 amino acids), and they have proved amenable to molecular manipulation/modification to enhance specific activities. These peptides are ideal as structural scaffolds for the production of unique new biologically active molecular species with highly specific targets or actions. Although of resultant great interest to the pharmaceutical industry, the problem of successful delivery of peptide therapeutics (or, potentially, offensive agents) remains an issue. This problem applies equally to the peptide toxins with different sites and modes of action, which also is the subject of pharmaceutical discovery research. For example, the diverse conopeptides are typically low molecular mass (<5 kDa) peptides that have complex pharmacology. More than a dozen classes of conotoxins exist, including:

- inhibitors or activators of voltage-gated sodium channels,
- nicotinic acetylcholine receptor inhibitors, and
- serotonergic 5-HT3 antagonists and many others.[47,48]

Some of these already have proven uses as pharmacotherapeutics. One conopeptide, ziconotide (Prialt, Jazz Pharmaceuticals, Dublin, Ireland), is already used as an alternative analgesic to treat moderate to severe refractory pain. Ziconotide, which is the only intrathecal nonopioid analgesic approved by the US Food and Drug Administration, binds to N-type calcium channels on primary nociceptive afferent neurons in the dorsal horn of the spinal cord.[49]

Some excitatory neurotoxins are larger polypeptides, and most of these, like the small pepide atracotoxins, stimulate neuroexocytosis. For example, the 110- to 140-kDa α-latrotoxins from widow spider (*Latrodectus* spp) venoms bind to several target proteins (eg, soluble *N*-ethylmaleimide-sensitive factor activating protein receptor [SNARE] proteins) in the neurolemma, and they cause calcium and sodium influx that results in massive neurotransmitter release.[50]

Clinically, these toxins cause symptoms and signs that reflect hyperexcitation of the nervous system, manifesting clinical effects, such as muscle fasciculations and spasms, tachycardia, paraesthesia, and more.[34,35,43,51] The pharmacology of these toxins may have broad similarities across many taxa of venomous animals, although with sometimes distinctive specific effects in particular groups.

Of the approximately 1,800 species of scorpions, only about 30 to 35 taxa are known to be dangerously venomous to humans. Most of these medically important species belong to the family *Buthidae*, and are widely distributed globally. The venom delivery apparatus (sting, the aculeus) is located at the end of the telson and delivers a bolus of venom. Many scorpions typically insert the sting into prey with protracted penetration. Stings delivered to potential predators or unfortunate humans can be relatively brief, but still may cause life-threatening effects, especially in children.

Medically dangerous buthid scorpions (Figure 18-11) often have numerous neurotoxic isotoxins present in their venoms. Some scorpion venoms contain long-chain polypeptide neurotoxins that act by stabilizing the open conformation of voltage-dependent sodium channels, thereby causing continuous and prolonged



Figure 18-11. The medically important scorpion, *Androctonus australis* (thick or fat-tailed scorpion, family *Buthidae*), is one of the most toxic species studied. This species is extensively distributed from Asia through the Middle East and northern Africa.
Photograph: Copyright © Julian White. In: White J. Venomous animals: clinical toxinology. *EXS*. 2010;100:233–291. In: Luch A (ed). *Molecular, Clinical and Environmental Toxicology*. Vol 2: *Clinical Toxicology*. Basel, Switzerland: Birkhäuser; 2010.

firing of the somatic, sympathetic, and parasympathetic neurons. The repetitive firing results in autonomic and neuromuscular hyperexcitation. Many of these toxins also stimulate neuroexocytosis. Well over 100 α-neurotoxins have been characterized from various scorpion venoms and have often been classified as toxic to mammals, insects, or both.[52] Interestingly, computational analyses of structure–function have suggested that scorpion α-toxins possess modular organization, and individual modules interact with different parts of their target sodium channels.[53] Most of these toxins have molecular mass around 65 kDa, share a common βαββ organization, contain four disulfide bridges, and primarily bind to site 3 of the voltage-gated sodium channel, thereby delaying inactivation; whereas other scorpion venom toxins (β-toxins) shift the membrane potential dependence of channel activation by binding to site 4.[54]

Some of the short polypeptide neurotoxins present in the same venoms simultaneously antagonize potassium channels. Most of these consist of 23 to 64 amino acids with molecular mass less than 4 kDa, and these structurally constrained polypeptides adhere to either the inhibitor cysteine knot or disulfide-directed β-hairpin folding motif.[55,56] The first structurally elucidated bound toxin-potassium channel complex contains the well-studied potassium channel-binding toxin charybdotoxin, isolated from venom of the Israeli yellow scorpion, *Leiurus quinquestriatus hebraeus*.[57] This imaginatively named distinctive toxin has specificity for a single site on the external end of big potassium channels, a form of Ca^{2+}-dependent, voltage-dependent K^+ channel that facilitates the passive flow of a relatively large current of potassium ions.[58,59] (The toxin was named after Charybdis, the daughter of Poseidon, who was transformed into a marine behemoth by Zeus. The whirlpools produced by the mythic Charybdis were analogized with the figuratively turbulent external face of the big potassium channel.[58,59] Other medically important scorpions often have been named with similar mythological flair. The toxic South American scorpion, *Tityus serrulatus* [Brazilian yellow scorpion, family *Buthidae*] derives its genus name from the giant, Tityus, who, according to Greek mythology, was banished to Hades by Zeus because of the former's attempt to rape his bride, the goddess Leto, a female Titan.) Charybdotoxin essentially "plugs" the channel closed, thereby antagonizing the flow of potassium current. Na^+ channel and K^+ channel toxins in scorpion venoms function synergistically, causing persistent depolarization of autonomic nerves and resulting in the massive release of autonomic neurotransmitters.[60]

Although the actual frequency and case morbidity/mortality rates of scorpion stings are unknown, at least several thousand fatalities occur per year. A recent global estimate suggested 1.2 million scorpion stings occur annually, with about 3,250 deaths (case fatality rate around 0.27%).[61] The hallmark features of envenoming are instant and severe local pain at the sting site, followed by rapid onset (usually within 15 to 60 minutes) of systemic envenoming that may include generalized pain, cardiac dysfunction, profuse sweating, labile blood pressure (hypertension or hypotension), and pulmonary edema. After envenoming by some members of the genus *Centruroides* spp (Central America, southwestern United States, and Mexico), bizarre signs such as rotational nystagmus may be seen in children. The cardiac dysfunction or pulmonary edema may prove fatal, especially in children.

Envenoming by Australian funnel-web spiders (*Atrax* spp and *Hadronyche* spp) produces a similar pattern of rapid hyperexcitation, starting with perioral parasthesiae and tongue fasciculation. These initial effects can progress in minutes to include hypertension, excessive sweating, salivation, lacrimation, tachycardia, pulmonary edema, hypoxia, coma, and death. If the envenomed victim survives this stage, they may develop progressive muscle fasciculation, bradycardia, hypotension, and terminal cardiac collapse. The envenoming syndrome has been likened to a catecholamine storm.



Figure 18-12. Brazilian banana spider (*Phoneutria nigriventer*). A medically important South American species of aeranomorph.
Photograph: Wikipedia Commons, public domain. https://commons.wikimedia.org/wiki/File:Wandering_spider.jpg.

Widow spiders (family *Theridiidae*, genus *Latrodectus*; approximately 31 species, including well-known species such as black widows [*Latrodectus mactans*] and red-backed spiders [*Latrodectus hasselti*; see Figure 18-2]) cause less rapid or severe envenoming, with initial bite site pain, and sometimes with local sweating and piloerection. Regional pain and sweating that progresses in severe cases to generalized pain, sweating, hypertension, nausea, and malaise may follow. This syndrome (latrodectism) is unpleasant and may last many hours to days, but is rarely fatal. Brazilian banana spiders (family *Ctenidae*, genus *Phoneutria*; approximately eight species; Figure 18-12) cause similar clinical effects (phoneutrism), but with more prominent local pain; additionally, envenomation in young males may result in priapism.

Australian Irukandji jellyfish (several genera of the class Cubozoa) may also cause a catecholamine storm-like envenoming. Irukandji syndrome is named for the Aboriginal clan in Cairns, Queensland, Australia, where this type of envenoming was first noted. The agent responsible for these cases remained elusive for years and eventually was determined to be a single cubozoan species, the chirodropid, *Carukia barnesi*, named for Dr Jack Barnes, who in 1964 first associated this jellyfish species with the syndrome. It is now clear that at least two species of chirodropid [*C barnesi*, *Malo kingi*], one or more species of cubozoan carybdeid [*Carybdea* spp], and probably several others are responsible for these serious envenomings. The victim initially experiences minor local sting effects and then a delayed (usually 20 to 40 minutes) onset of systemic envenoming with severe muscle spasm pain (especially in the back), sweating, nausea, and hypertension that can fluctuate and recur over hours. In severe cases, cardiac dysfunction and pulmonary edema can occur, and although deaths have occurred, they are rare (see Table 18-2). Still, any patient presenting with a significant Irukandji envenoming should have continuous cardiac monitoring.

Antivenoms are available for some medically important scorpion species and, despite some controversy, the majority of evidence indicates that these are effective and lifesaving if administered intravenously at the earliest opportunity. Some information has suggested that glucocorticoids might improve outcomes in some severe scorpion envenoming cases. However, these suggestions have largely been based on low quality evidence, and a recent Tunisian case-control study found no benefit.[62,63]

Antivenom against Australian funnel-web spiders is highly effective and can be lifesaving. Antivenom for widow spider bites is used routinely in Australia, where most clinical practice suggests it is effective, or at least more effective than alternative therapies. Elsewhere, its use is often reserved only for the most severe cases, mostly due to largely overrated fears of side effects that are actually uncommon.

A similar situation exists for *Phoneutria* bites, where local pain relief, including local anesthesia, is used in preference to antivenom, except in children and the most severely envenomed. No antivenom for Irukandji stings exists, and treatment is supportive and includes use of opioid analgesia to help alleviate the severe systemic pain associated with serious Irukandji envenoming.

Myotoxins

Myotoxins are mostly systemic in action and commonly are PLA2, but some myotoxins, such as the 42 amino acid basic polypeptide crotamine, appear to have focused action on muscle groups in the lower extremities. Crotamine, which is found in selected North American rattlesnake venoms, probably aids prey capture by causing rear limb dysfunction, that inhibits locomotor ability (eg, prevention of lengthy prey travel post-envenoming). Hypothetically, it may decrease the metabolic cost of trailing envenomed prey (eg, following bite-release, an envenomed prey animal may not expire for a few minutes and have time to flee from the site of the encounter with the snake). Locally acting myotoxins cause cellular damage around the bite site, but the systemically acting myotoxins, particularly PLA2, selectively target

skeletal muscle and can cause extensive and severe muscle damage. Although binding may occur early, once venom has reached and then exited the circulation, a delay occurs before onset of clinical detection of pathology. Therefore, significant myolytic effects may occur before clinical indicators for treating myolysis, which has led some researchers to suggest that early intervention with antivenom is justified to prevent myolysis in some cases of bites inflicted by species known to produce serious myolytic effects.[64] This remains to be studied, as no current evidence supports this approach.

The systemic myotoxins bind to the skeletal muscle cells and cause progressive and severe damage to the cells. Experimental animals injected with purified myotoxic PLA2 often exhibit skeletal muscle changes characterized by dissolution of actin and myosin filaments, disruption of Z-band material, dilation of the sarcoplasmic reticulum, and swelling and disruption of mitochondria, as well as disorganization of the T-tubule system.[65–68] However, if the basement membrane is preserved, some muscle regeneration can occur that may commence 24+ hours postbite, but may require weeks to complete. It is thought that the PLA2 enzymatic action is a crucial component in their toxicity, but chemical modification of specific residues (eg, Asp49, Lys49, and others) in the primary sequence of some of these enzymes has suggested that some PLA2 species may have a pharmacologically active domain discrete from the catalytic functional site.[69] Clearly, cellular binding to the target cells is an essential first step, as the myotoxic PLA2 species do not cause widespread cellular injury and specifically target muscle cells.



Figure 18-14. Australian eastern brown snake (*Pseudonaja textilis*). The most medically important snake in Australia; its range encompasses a large proportion of Australia and southeastern Papua New Guinea. Its venom contains potent procoagulants and a presynaptic neurotoxin (textilotoxin) with the highest experimental lethal potency of any snake venom toxin isolated to date. Fortunately, many bites inflicted on human victims are dry, meaning no venom is injected. Photograph: Copyright © Julian White. In: Brent J, Wallace KL, Burkhart KK, et al (eds). *Critical Care Toxicology: Diagnosis and Management of the Critically Poisoned Patient*. 1st ed. Philadelphia, PA: Mosby; 2005.



Figure 18-13. Australian common tiger snake (*Notechis scutatus*). One of the world's most venomous snakes, its venom contains potent presynaptic neurotoxins, myotoxins, and procoagulants.
Photograph: Copyright © Julian White. In: White J. *A Clinician's Guide to Australian Venomous Bites and Stings*. Melbourne, Australia: Commonwealth Serum Laboratories; 2013: 300 pp.

PLA2 myotoxins or multimeric toxins containing PLA2 subunits (many with myolytic activity; see Table 18-2 for representative examples) are found principally in snake venoms, notably selected Australian elapid venoms (eg, notexin [common tiger snake, *Notechis scutatus*; Figure 18-13], textilotoxin [Eastern brown snake, *Pseudonaja textilis*; Figure 18-14], taipoxin [coastal taipan (*Oxyuranus scutellatus*)]), sea snake venoms [eg, myotoxin VI5 (beaked sea snake, *Hydrophis* [*Enhydrina*] *schistosus*[70]], PLA2-H1 [blue-banded or annulated sea snake, *Hydrophis cyanocinctus*]), and some krait venoms (eg, β-bungarotoxin found in several species [eg, *Bungarus candidus*; Figure 18-15]). They are also found in several rattlesnake (family *Viperidae*, subfamily *Crotalinae*) venoms (eg, crotoxin, found in venoms of several taxa of tropical rattlesnakes, including *C durissus* spp, and others such as Mojave toxin and its isotoxins in venom of the Mojave rattlesnake, *C scutulatus*, tiger rattlesnake, *C tigris* [Figure 18-16], timber or canebrake rattlesnake, *C horridus* and others), as well as some Russell's viper (family *Viperidae*, subfamily *Viperinae*) venoms (eg, possibly, VRV-PL4 [*D russelii*; Figure 18-17]).



Figure 18-15. Malayan or blue krait (*Bungarus candidus*), Thailand. A semi-fossorial species that ranges in Malaysia, parts of Southeast Asia, and Indonesia. Studied *Bungarus* spp have venoms containing highly potent presynaptic neurotoxins (bungarotoxins), postsynaptic neurotoxins, and other components, including some with cardiotoxic properties.
Photograph: Copyright © Julian White. In: Brent J, Wallace KL, Burkhart KK, et al (eds). *Critical Care Toxicology: Diagnosis and Management of the Critically Poisoned Patient*. 1st ed. Philadelphia, PA: Mosby; 2005.



Figure 18-16. Tiger rattlesnake (*Crotalus tigris*). This distinctive rattlesnake has venom that contains the crotoxin homologue, Mojave toxin, a potent presynaptic heterodimeric neurotoxin. There are only a few documented bites by this taxon, all of which were medically insignificant. However, any rattlesnake species with venom that contains Mojave toxin, crotoxin or related isotoxins (eg, horridus toxin, or canebrake toxin found in venom of some geographic populations of the timber rattlesnake, *C horridus*) must be considered capable of delivering a potentially fatal envenomation.
Photograph: Copyright © Julian White. In: White J, Dart RC. *Snakebite: A Brief Medical Guide*. Denver, CO: Rocky Mountain Poison & Drug Center; 2008.

Clinically, systemic myotoxicity presents several to many hours postbite as muscle pain, muscle weakness, myoglobinuria, and gross elevation of plasma creatine phosphokinase and often-raised hepatic enzymes (eg, alanine transaminase, alkaline phosphatase, aspartate aminotransferase). In some cases, a notable creatine phosphokinase elevation may be observed before muscle discomfort.

It is important to note that some snake species often considered as purely neurotoxic may inflict bites that can cause mixed neurotoxicity and rhabdomyolysis akin to that seen in some Australian elapid envenoming by coastal taipans, tiger snakes, and others. For example, bites from greater black kraits (*Bungarus niger*) and Malayan kraits (*Bungarus candidus*; see Figure 18-15) have respectively caused mixed neurotoxicity and myotoxicity in Bangladesh[71] and myotoxicity, cardiovascular instability, neurotoxicity, and hyponaetremia in southern Vietnam.[72] Thus, due to unpredictable venom variability, as well as clinical response to variable venom components, it is essential not to view a given species as solely "neurotoxic" or "hemotoxic," because the venom-induced disease may notably vary. Treatment is with early intravenous administration of antivenom and supportive treatment, especially to ensure good renal output with aggressive fluid resuscitation (as in any recommendation for clinical



Figure 18-17. Russell's viper (*Daboia russelii*), Bannerghatta, India. Along with the saw-scaled vipers (*Echis* spp) and several species of cobras (*Naja* spp), *D russelii* and *D siamensis* (Eastern Russell's viper) are the species most important in the global envenoming burden. The snakes have a wide distribution and are plentiful; they constitute a public health problem particularly among rural communities in the Indian subcontinent, as well as parts of Southeast Asia. Envenoming from *D russelii* from different geographic populations can result in several differing clinical syndromes including hypogonadism, one of the consequences of pituitary hemorrhagic infarct (Sheehan's syndrome) resulting in panhypopituitarism. Another member of the genus, *D palaestinae* (Palestine viper) is medically significant in the Middle East. Its venom has been studied as a source of several classes of pharmacotherapeutics, including analgesics.
Photograph: Wikipedia Commons, public domain. https://commons.wikimedia.org/wiki/Daboia_russelii#/media/File:Russellsviper_sal.jpg.

management, approaches such as aggressive fluid resuscitation must be applied in the setting of risk/benefit with consideration for the patient's possible preexisting comorbidities, such as congestive heart failure or other volume overload states).

With major myolysis, secondary renal failure is a risk and can contribute to severe and sometimes fatal hyperkalemic cardiac toxicity. Alkalinization of urine is sometimes recommended, but is unproven to provide any added benefit in snakebite myolysis, as well as in most other presentations (eg, serotonergic syndrome, crush injuries) featuring acute myoglobinuria capable of producing nephropathy.

Myolysis can also follow some other envenomings, including massive bee stings and ingestion of certain mushrooms (eg, family *Tricholomataceae*, *Tricholoma* [*flavovirens*] *equestre*, yellow knight mushroom).[73] Studies of some venomous fish have reported myotoxicity in the murine model,[74] but, to date, there are no well-documented clinical cases of fish stings having caused myotoxicity.

Hemostasis-Active Toxins

The complex human hemostatic system is a common target of venoms, particularly snake venoms, that may cause a wide variety of clinical effects most commonly associated with an increased bleeding tendency. When combined with the action of proteolytic hemorrhagins, some cause severe hemorrhagic diathesis, a pathological state that could hypothetically be viewed as inducing terror in some individuals or populations. Thus, although impractical as tactically deployed bioweapon agents, some of these toxins could conceivably be used offensively on a small scale. An overview of the major toxin groups involved is shown in Table 18-3.

Venoms cause activation or inhibition of the clotting system by several different mechanisms and at many possible target points (Figure 18-18). Some venoms contain multiple toxins affecting hemostasis that may be synergistic, independent, or counteracting (see Figure 18-18).

In most cases, this type of envenoming (most accurately termed coagulopathic, not hemotoxic) causes an increased bleeding tendency, often by either hydrolyzing clotting protein (fibrinogenases) or by activating normal systems of clot formation and dissolution (procoagulants). These activities are most widely represented in viperid snake venoms, and a prominent example is that of ecarin, the prothrombin-converting metalloprotease (and closely related toxins) found in saw-scaled viper (*Echis* spp; Figure 18-19) venoms (a group A prothrombin activator). Ecarin plays an important role in the clinical laboratory by providing a meizothrombin generation test allowing for the precise quantification of direct thrombin inhibitors. Russell's viper (*Daboia russelii*) venom is a pivotal reagent in laboratory medicine because it contains a phospholipid and Ca^{2+}-dependent potent activator of factor X that forms a complex with prothrombin and thereby converts fibrinogen to fibrin. This mechanism facilitates the diagnosis of phospholipid antibodies including lupus anticoagulant, because in these states the antibodies bind to the essential venom co-factor phospholipid and thus inhibit the venom-induced factor X activation and prolong the clotting time.

Some Australian elapids also have potent procoagulant venoms. A number of the procoagulants present in these venoms, such as those found in the common tiger snake and Eastern brown snake, have structures homologous to human clotting factors Xa (group D prothrombin activator) or Va and Xa combined (group C prothrombin activator). Other animals, such as the venomous lizards *Heloderma suspectum* (Gila monster) and *Heloderma horridum* (beaded lizard), have venoms that contain procoagulant toxins and other hemostasis-active toxins, such as the *H horridum* venom acidic PLA2 that inhibits thromboxane-induced platelet aggregation.[75] As mentioned previously, caterpillars of the Brazilian *saturniid* moth, *Lonomia*, have irritating hairs that contain toxins with powerful procoagulant effects. Envenoming by a caterpillar may seem far-fetched, but it is not to be taken lightly because *Lonomia* stings can produce fatal disseminated intravascular coagulopathy in humans. A few animals, such as the Martinique lancehead viper or fer-de-lance *Bothrops lanceolatus*, have prothrombotic venom, and envenoming can lead to deep venous thrombosis, pulmonary emboli, cerebral infarction, and related thrombotic events.

Clinically, toxins promoting increased bleeding tendency may cause few initial or early symptoms. In many cases, almost all circulating fibrinogen can be consumed without apparent bleeding until or unless bleeding is induced through injury or a medical procedure such as venipuncture. In the former case, a fall with relatively mild cranial trauma can result in catastrophic intracranial bleeding that may occur minutes to hours later.

To detect abnormalities at the earliest opportunity after a potentially coagulopathic bite or sting, serial laboratory assessment of clotting function is essential. Careful serial laboratory testing is a cornerstone of management because early provision of antivenom and other potential treatments (eg, replacement therapy; see next paragraph) can limit the possible cardiovascular effects induced by the venom disease. There is ongoing controversy over the role of antivenom in

TABLE 18-3

MAJOR VENOM TOXIN GROUPS AFFECTING HUMAN HEMOSTASIS

Toxin Type	Effect	Examples
Procoagulants	Factor V activating Factor X activating Factor IX activating Factor II (prothrombin) activating: Group A Group B Group C Group D Fibrinogen clotting	RVV-V (Factor V-activating serine protease from venom of the Russell's viper, *Daboia russelii*) Contortrixobin (Factor V activating serine protease from venom of the copperhead, *Agkistrodon contortrix*) RVV-X (metalloproteinase disintegrin activator of Factor X from venom of the Russell's viper) TSV-FIX-BP (C-type lection-activating Factor IX from venom of Stejneger's green tree viper, *Trimeresurus stejnegeri*) Ecarin (cofactor-independent metalloproteinase Group A prothrombin activator from venom of the saw-scaled viper, *Echis carinatus*) Carinactivase (Ca^{2+}-dependent metalloproteinase Group B prothrombin activator from venom of the saw-scaled viper) Oscutarin (Ca^{2+}- and phospholipid-dependent serine protease Group C prothrombin activator from venom of the coastal taipan, *Oxyuranus scutellatus*) Notecarin (Ca^{2+}-, phospholipid, and Factor Va-dependent serine protease Group D prothrombin activator from venom of the common tiger snake, *Notechis scutatus*)
Anticoagulants	Protein C activating Factor IX/X activating protein Thrombin inhibitor Phospholipase A2	ACC-C (protein C-activating serine protease from venom of the copperhead, *Agkistrodon controtrix*) Bothrojaracin (C-type lectin thrombin inhibitor from venom of the jararaca, *Bothrops jararaca*) CM-IV (anticoagulant PLA2 from venom of the African black-necked spitting cobra, *Naja nigricollis*)
Fibrinolytic	Fibrin(ogen) degradation Plasminogen activation	Ancrod (fibrinogenolytic enzyme from venom of Malayan pit viper, *Calloselasma rhodostoma*) Neuwiedase (α-chain fibrinogenase [metalloproteinase] from venom of the jararaca pintada, *Bothrops neuwiedi*) Brevinase (β-chain fibrinogenase (serine protease) from venom of the mamushi, *Gloydius blomhoffi*) TSV-PA (plasminogen-activating serine protease from venom of Stejnegers green tree viper, *Trimeresurus stejnegeri*)
Vessel wall interactive	Hemorrhagins	Echistatin (RGD disintegrin from venom of the saw-scaled viper, *Echis sochureki*)
Platelet activity	Platelet aggregation inducers Platelet activation inhibitors	Botrocetin (platelet agglutination with VWF from venom of the jararaca) Convulxin (C-type lectin platelet aggregation, from venom of the South American rattlesnake, *Crotalus durissus terrificus*) Jararhagin (RGD disintegrin snake venom metalloproteinase that causes inhibition of platelet aggregation, from venom of the jararaca) Echicetin (C-type lectin platelet aggregation inhibitor from venom of the saw-scaled viper)
Plasma protein activators	Serine protease inhibitors	Proteinase I and II (inhibition of serine protease inhibitors from venom of the eastern diamondback rattlesnake, *Crotalus adamanteus*)

RGD: arginine-glycine-aspartic acid, a peptide motif found in this group of snake venom metalloproteinases; vWF: Von Willebrand Factor
Data sources: (1) Mebs D. *Venomous and Poisonous Animals: A Handbook for Biologists, Toxicologists and Toxinologists, Physicians and Pharmacists*. Boca Raton, FL: CRC Press; 2002: 360. (2) Meier J, White J (eds). *Handbook of Clinical Toxicology of Animal Venoms and Poisons*. Boca Raton, FL: CRC Press; 1995. (3) Mackessy SP (ed). *Handbook of Venoms and Toxins of Reptiles*. Boca Raton, FL: CRC Press, Taylor & Francis; 2010: 528 pp. (4) Synthesized information included in lectures and presentations of the authors.

treating snakebite-induced coagulopathy in Australia, but most authorities consider timely administration of antivenom as the optimal treatment. Outside Australia, antivenom remains the evidence-based treatment for snakebite coagulopathy, either for increased bleeding (eg, from bites by the saw-scaled vipers, *Echis* spp) or for increased clotting (eg, from bites by the Martinique lancehead viper or fer-de-lance *B lanceolatus*).

The role of blood clotting products (eg, fresh frozen plasma and cryoprecipitate) replacement remains controversial, and when antivenom is available, it should be used in preference to and before such blood products if the clinical circumstances (eg, severe depletion with notable bleeding risk) suggest the need for replacement. For patients with major bleeding, despite adequate antivenom, blood products may have a place as adjunctive treatment depending on clinical need. Anticoagulant drugs such as heparin and warfarin are not useful in treating snakebite coagulopathy, probably intensify bleeding, and are positively contraindicated.

Hemorrhagic and Hemolytic Toxins

Some snakes have venom toxins that actively damage blood vessels and other tissues, thereby promoting bleeding. When combined with pro- and/or anticoagulant toxins, the actions are synergistic and potentially cause extensive bleeding or tissue injury, particularly around the bite site. These toxins are often also called hemotoxins, but are more accurately termed vasculotoxins because of their direct effects on the microvasculature.

Most hemorrhagic toxins are metalloproteinase enzymes, usually with a zinc moiety. However, some are comprised of peptide complexes, such as the synergistically hemorrhagic PLA2-peptide complex (DR-HC-1), characterized from *D russelii* venom.[76] The larger venom metalloproteinases have additional domains carboxy to the zinc-binding domain. Some metalloproteinases, termed class P-II by many investigators, contain domains that are further processed and give rise to free domains such as disintegrins.[77–79] Class P-III metalloproteinases have disintegrin-like and cysteine-rich domains, whereas class P-IV is similar to P-III, but its metalloproteinases have additional lectin-like domains. Homologs of the venom P-III structures (ADAMs: A Disintegrin-Like And Metalloproteinase-containing protein) have been identified in a variety of mammalian sources and tissues[77] and possess myriad activities, including participation in essential cellular functions such as angiogenesis regulation, inflammation, matrix protein processing, and many others.[80] However, particular focus has been directed toward those that occur in reptile venoms and mammalian reproductive tissues (the reprolysins, or M12 metalloproteinase subfamily[79]). The angiogenic inhibitor and cell adhesion molecule regulation functions have attracted scrutiny of some of the venom disintegrins as potential antineoplastic agents.[81] In addition, tissue inhibitors of metalloproteinases not only regulate proteinases in mammalian systems, but also have signal-transduction roles that continue to be characterized.[82] This is a fertile and promising area of research, and full consideration of their structure-function is beyond the scope of this chapter.

The proteolytic actions of these toxins damage the endothelium/basement membrane of cells comprising the microvasculature. Some crotaline viperids such as the Western diamondback rattlesnake have venoms that contain several different isoforms (eg, atrolysins) of these toxins. Some hemorrhagins have a shared disintegrin domain (a 13-amino acid loop containing specific sequences of arginine, glycine, and aspartic acid, or RGD in abbreviated nomenclature) that facilitates binding to platelet receptor gIIb/IIIa. These toxins have attracted more attention than most snake venom hemorrhagins because of their use in integrin function studies[83] and for their roles as structure-function scaffolds for pharmacotherapeutics (especially for the development of antithrombotics and as antineoplastics). One of these peptides, barbourin, has been characterized from venom of the dusky pygmy rattlesnake (*Sistrurus miliarius barbouri*).

Clinically, these hemorrhagins contribute to rapid and potentially severe local tissue effects such as blistering, bleeding, and necrosis, which are mostly associated with significant local pain and sometimes major fluid shifts from the circulation into local tissues. This latter effect can result in profound hypovolemic shock. Although some characterized hemorrhagins, such as the 50 kDa nonproteolytic hemorrhagin from venom of *Atractaspis engaddensis* (Israeli or oasis mole viper or burrowing asp), exhibit dose-related hemorrhagic activity, others such as the atrolysins mentioned previously may induce dose-independent hemorrhage in vivo as a result of the combined activities of this rhexic hemorrhagin and other venom components, such as the 30 kDa antagonist that binds to platelet glycoprotein receptor Ib. In this instance, the inhibition of platelet adhesion probably acts synergistically with the hemorrhagins and other anticoagulant toxins, thereby increasing the hemorrhagic effect to a greater extent than that accomplished by any of the single components alone.

The role of antivenom in treating effects of hemorrhagins is controversial, although most authorities recommend using antivenom therapy for prohemorrhagic envenomings. However, the molecular size and steric hindrance posed by antibodies present in

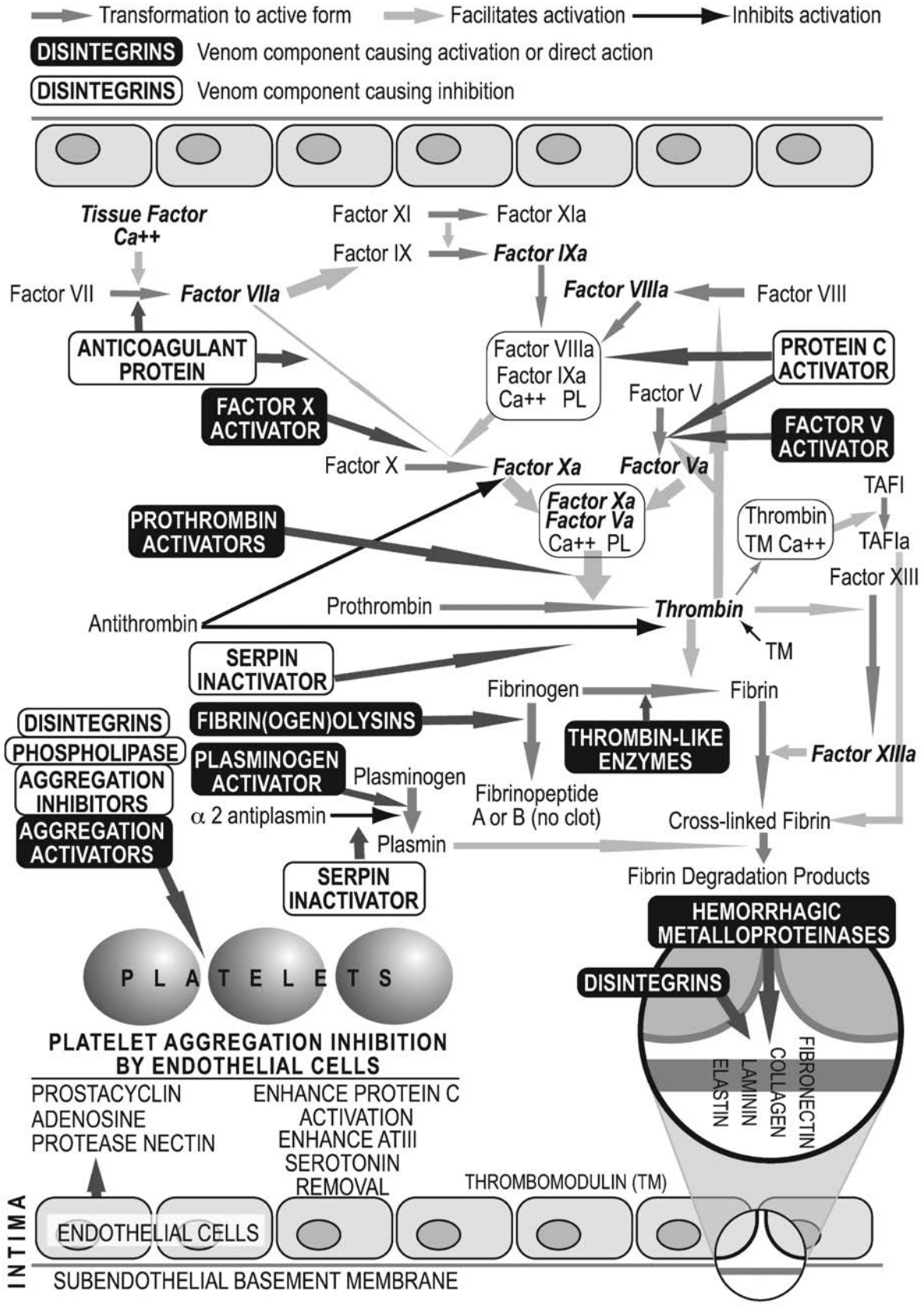

Transformation to active form
Facilitates activation
Inhibits activation
DISINTEGRINS
Venom component causing activation or direct action
DISINTEGRINS
Venom component causing inhibition
Tissue Factor
Ca++
Factor XI
Factor XIa
Factor IX
Factor IXa
Factor VII
Factor VIIa
Factor VIIIa
Factor VIII
ANTICOAGULANT PROTEIN
Factor VIIIa
Factor IXa
Ca++ PL
PROTEIN C ACTIVATOR
Factor V
FACTOR X ACTIVATOR
FACTOR V ACTIVATOR
Factor X
Factor Xa
Factor Va
Factor Xa
Factor Va
Ca++ PL
TAFI
PROTHROMBIN ACTIVATORS
Thrombin
TM Ca++
TAFIa
Factor XIII
Antithrombin
Prothrombin
Thrombin
TM
SERPIN INACTIVATOR
Fibrinogen
Fibrin
DISINTEGRINS
FIBRIN(OGEN)OLYSINS
THROMBIN-LIKE ENZYMES
Factor XIIIa
PHOSPHOLIPASE
PLASMINOGEN ACTIVATOR
Plasminogen
AGGREGATION INHIBITORS
Fibrinopeptide A or B (no clot)
α 2 antiplasmin
Cross-linked Fibrin
AGGREGATION ACTIVATORS
Plasmin
SERPIN INACTIVATOR
Fibrin Degradation Products
HEMORRHAGIC METALLOPROTEINASES
DISINTEGRINS
PLATELETS
FIBRONECTIN
COLLAGEN
LAMININ
ELASTIN
PLATELET AGGREGATION INHIBITION BY ENDOTHELIAL CELLS
PROSTACYCLIN
ADENOSINE
PROTEASE NECTIN
ENHANCE PROTEIN C ACTIVATION
ENHANCE ATIII
SEROTONIN REMOVAL
THROMBOMODULIN (TM)
INTIMA
ENDOTHELIAL CELLS
SUBENDOTHELIAL BASEMENT MEMBRANE

Figure 18-18. Diagrammatic representation of the human hemostasis system, with examples of where venoms may interact to activate or inhibit the clotting process. The diagram only represents some key elements of hemostasis, not every component currently known and similarly, only some common examples of toxin effects, not an exhaustive listing of all known toxin interaction sites. The diagram concentrates particularly on the coagulation cascade elements, whereby various triggers in normal hemostasis activate top levels of the cascade, resulting in progressive activation downstream until common pathways are reached. Principal among the latter are activation of procoagulant enzymes (mostly serine proteases), such as factor VIII to FVIIIa, factor X to FXa, factor V to FVa, with FXa and FVa forming the prothrombinase complex that then activates the final enzymatic step, prothrombin (factor II) to thrombin (FIIa). It is thrombin that then converts the clotting protein, fibrinogen, to fibrin, so that fibrin molecules can be cross-linked by factor XIIIa, to form a stable fibrin clot. Normally this clot formation occurs inside a protective platelet plug environment, at the site of blood leakage from a damaged blood vessel. The damaged edges (endothelial cells) of the blood vessel are a potent stimulus to activation of the hemostasis system. The fibrin blood clot then provides a semipermanent plug to prevent further blood leakage from the damaged blood vessel, allowing time (6–10 days) for the endothelium to repair and effect a permanent repair of the damage, at which time the fibrin clot is no longer required and is broken down by plasmin (activated form of plasminogen). There are a number of promoters and inhibitors of each step in this complex multistep process, each designed to ensure clots only form where needed and are dissolved once no longer needed. Each and every step in the process, including promoters and inhibitors, is a potential target for venom toxins. In addition, if the process is activated freely in blood, rather than in a protected platelet plug environment, some normal controls are bypassed, allowing rapid progression from procoagulant activation, through conversion of fibrinogen to fibrin, and then degradation of fibrin strands and cross-linked fibrin by plasmin. This process can potentially cause rapid consumption of all circulating or available fibrinogen, rendering the patient unable to form blood clots, so at risk of major bleeding. In one sense this is a profound form of anticoagulation, but it is achieved by aggressive stimulation of clotting, hence is usually termed a *procoagulant defibrination coagulopathy* (referred to as VICC by a few authors). This consumptive coagulopathy can be initiated by a variety of venom toxins, particularly the calcium-independent procoagulants (Groups A and B) found in saw-scaled viper (*Echis* spp) venoms, and the calcium-dependent procoagulants (Groups C and D) found in many Australian Elapid snake venoms (eg, *Pseudonaja* spp, *Oxyuranus* spp, *Notechis* spp, *Tropidechis carinatus*, *Hoplocephalus* spp). These latter procoagulants mimic closely the normal clotting factors, thus have extensive homology with either FXa, or the FVa–FXa (prothrombinase) complex. It can be speculated that these toxins were developed from recruitment of normal clotting factor genes. Once this process is activated there can be massive consumption of clotting factors, especially fibrinogen, but also FII, FV, FVIII, and FX among others, and while antivenom may, at least for some venoms, switch off the process, it cannot instantly restore depleted fibrinogen and other factor levels, so several hours may pass before protective levels are reached. Although giving factor replacement therapy (fresh frozen plasma, cryoprecipitate, or whole blood) may speed return to normal levels, if neutralization of venom toxins is incomplete, such replacement therapy can instead add fuel to the coagulopathy fire, potentially worsening the clinical picture. In contrast, phospholipase A2-based anticoagulant toxins (eg, from *Pseudechis* spp) merely inhibit portions of the hemostasis pathways without causing consumption of clotting factors, so that antivenom can almost instantly reverse this effect.
PL: phospholipid; TAFI: thrombin-activatable fibrinolysis inhibitor; VICC: venom-induced consumption coagulopathy
Photograph: Copyright © Julian White. In: White J. Venomous animals: clinical toxinology. *EXS*. 2010;100:233–291. In: Luch A (ed). *Molecular, Clinical and Environmental Toxicology*. Vol 2: *Clinical Toxicology*. Basel, Switzerland: Birkhäuser; 2010.

antivenom (usually whole IgG or the F(ab')^2 portion of IgG) may not easily leave the circulation to penetrate the areas being damaged by hemorrhagins. Drugs to directly target the enzymatic action of these toxins have been explored as adjunctive therapy and continue to attract study to identify possible alternative therapeutic agents.

In cases of hemorrhagic snakebite, it is essential to monitor for shock and ensure adequate intravenous hydration. The damaged bite area may appear markedly swollen and with poor vascular return, with concomitant pain, possibly suggesting an underlying compartment syndrome. Although snakebites may rarely cause compartment syndrome, most snakebite patients do not have definitive evidence of compartment syndrome. In addition, the clinical criteria defining compartment syndrome varies among surgical specialties (eg, orthopedics and vascular surgery), facilities, and individual physicians and surgeons. In facilities lacking intracompartmental measurement catheter systems, clinically experienced use of basic Doppler ultrasound may help clinical interpretation. Conversely, hospitals equipped with magnetic resonance imaging or multidetector computed tomographic arteriography could provide additional means to assess complicated cases, but the latter method would be positively contraindicated in any patient with coagulopathy.

Fasciotomy is the standard treatment for compartment syndrome, but when used injudiciously for snakebite it can accelerate blood loss and shock, and cause severe permanent tissue injury. Fasciotomy should only be performed in snakebite if there is direct pressure measurement confirmation of compartment syndrome with clinical correlation and then only after any coagulopathy has been treated. Several patients of



Figure 18-19. Northeast African saw-scaled or carpet viper (*Echis pyrimidum*). There are approximately 11 current recognized species of *Echis*. Several *Echis* spp cause a large proportion of the world's human envenoming burden, and are among the three most medically important venomous snakes. Conversely, *E carinatus* venom has yielded components important in laboratory medicine (eg, the metalloprotease prothrombin activator, ecarin), as well as a platelet aggregation inhibitor (tirofiban, marketed as Aggrastat) that reversibly binds to platelet GPIIb/IIIa receptors.
GP: glycoprotein
Photograph: Copyright © Julian White. In: Brent J, Wallace KL, Burkhart KK, et al (eds). *Critical Care Toxicology: Diagnosis and Management of the Critically Poisoned Patient*. 1st ed. Philadelphia, PA: Mosby; 2005.

snakebite-induced, verified compartment syndrome have also been treated successfully by purely medical methods (eg, hyperbaric oxygen therapy and cautious use of mannitol, the latter contraindicated in cases featuring hypovolemia).

Cardiotoxins

True cardiotoxins that cause direct cardiac effects in humans are uncommon. Some examples include the following:

- the 2.5 kDa endothelin homologues, the sarafotoxins from some venoms of the mole vipers or burrowing asps (*Atractaspis* spp; these are not vipers at all, but instead they are front-fanged members of the subfamily *Atractaspinae* of the African family *Lamprophiidae*),
- bufotoxins from toad (eg, the marine toad, *Rhinella marina Linnaeus* 1758, formerly *Bufo marinus*) parotid gland secretions, and
- the oleandrins from oleander plants (*Nerium oleander* [oleander; Figure 18-20] and *Cascabela* (*Thevetia*) *peruviana* [yellow oleander or lucky nut; Figure 18-21]).

The cardiac glycosides of both of the latter plants produce effects similar to digoxin by binding to and inactivating the Na^+/K^+-ATPase pump on the cytoplasmic



Figure 18-20. Oleander (*Nerium oleander*) and yellow oleander or lucky nut (*Cascabella* [*Thevetia*] *peruviana*). These attractive and popular ornamental plants contain digoxin-like toxins that inactivate the Na^+/K^+-ATPase pump on the cytoplasmic membrane of myocardiocytes. Concentrations of the toxins present in the plant vary according to plant component (eg, seeds, leaves, etc), but ingestion of small amounts is potentially fatal. (**A**) Oleander (*Nerium oleander*). (**B**) Yellow oleander or lucky nut (*Cascabella [Thevetia] peruviana*).
Photographs: Courtesy of Julian White.



Figure 18-21. Indo-Chinese spitting cobra (*Naja siamensis*). This cobra is medically important throughout its range in Southeast Asia (Thailand, Lao, southern Vietnam and Cambodia), but its congener, *N kaouthia* (the monocellate, or monocled cobra) is responsible for a large proportion of snakebite mortality and morbidity throughout its extensive range in Southeast Asia and the Indian subcontinent. Envenoming commonly features serious local effects including desquamation, cellulitis and necrosis, and can include systemic neurotoxicity, as well as rare direct cardiotoxicity. Predominance of local envenoming or neurotoxicity is variable due to marked population venom variance, and some populations can inflict envenoming with mixed clinical presentation of local effects and neurotoxicity. In addition, *N siamensis* and probably several populations of *N kaouthia* can forcibly eject ("spit") venom through modified fangs that contain an anteriorly oriented orifice. The venom is targeted at the head of the recipient, and often enters the eyes causing venom ophthalmia. Agrarian-based rural communities may be seriously impacted by these snakes, as well as by *Echis* spp and *Daboia* spp, because farmers working sustenance crops often are the victims of envenomings by these species and, when not fatal, the common disabling sequelae can threaten their livelihood.
Data source: Chu ER, Weinstein SA, White J, Warrell DA. Venom ophthalmia caused by venoms of spitting elapids and other snakes: report of ten cases with review of epidemiology, clinical features, pathophysiology, and management. *Toxicon*. 2010;56:259–272.
Photograph: Copyright © Julian White. In: Brent J, Wallace KL, Burkhart KK, et al (eds). *Critical Care Toxicology: Diagnosis and Management of the Critically Poisoned Patient*. 1st ed. Philadelphia, PA: Mosby; 2005.

membrane of cardiac cells.[22] As a result, intracellular Na^+ concentration increases, which affects the Na^+/Ca^{2+} exchange channels, resulting in an increase in intracellular Ca^{2+} that leads to a positive inotropic effect. The increase of intracellular Ca^{2+} ions also raises the resting membrane potential of the cell, leading to increasing rates of spontaneous cellular depolarization and myocardial automaticity.[84] Inhibition of the Na^+/K^+-ATPase pump affects the intracellular movement of K^+ leading to hyperkalemia.[84,85]

A few reports show dysrhythmias/cardiotoxicity after cobra bites (especially the monocellate or monocled cobra, *Naja kaouthia*), and some of these probably are caused by the direct effects of cardiotoxin (also known as direct lytic factor due to its weakly hemolytic and other cytotoxic properties). Similar effects may be caused by secondary complications that might be more likely in victims with cardiovascular comorbidities (see Figure 18-21).[86,87]

Secondary cardiac effects are more common, such as the severe and sometimes lethal hyperkalaemia, secondary to severe myolysis that can follow secondary renal failure, which in some cases probably results from myoglobinuric nephropathy. The myolysis causes massive release of intracellular potassium into the circulation, which is controlled by kidney excretion, but with renal failure this normal kidney function is impaired or ceases, and potassium levels in blood subsequently rise to toxic levels. Some scorpion venom toxins cause neuroexcitation that can result in secondary cardiac dysfunction, sometimes with output failure, arrhythmias, and cardiogenic pulmonary edema. Cardiac arrhythmias and arrest may also be indirectly caused by hyperkalemia resulting from the cytotoxicity caused by high doses of toxins from box jellyfish (eg, the Australian box jellyfish or sea wasp, *Chironex fleckeri*) and the closely related multiple taxa (eg, *C barnesi* and *M kingi*) that cause Irukandji syndrome. The clinical effects of cardiotoxins depend on the toxin and its mechanisms of action.

Antidotes may be available for some "true," or more accurately, directly acting cardiotoxins. For digoxin-like toxins (such as bufonid toad poisoning or ingestion of oleander plants), cardiac effects can sometimes be reversed using anti-digoxin Fab' (digoxin fragment antibodies, or antigen-binding fragments). In one case of possible cobra envenoming-induced cardiotoxicity, antivenom administration appeared to correct ventricular bigeminy, possibly a result of the neutralization of toxin effects, or an improvement of other venom effects, thereby alleviating the secondary effects of envenoming.[87] For box jellyfish, although antivenom can neutralize the toxins, the indirect cardiovascular effects can develop so rapidly that, under experimental conditions using rodents, even giving antivenom together with the toxin does not prevent death. Nevertheless, prevailing opinion currently supports provision of high dose antivenom in cardiac collapse caused by box jellyfish stings, combined with aggressive cardiac resuscitation.

Nephrotoxins

Few direct animal venom or plant-derived nephrotoxins are currently known. Examples include toxins from Russell's viper venom and from several mushroom taxa: *Cortinarius* spp mushrooms (sometimes called cortinars or webcaps), *Amanita smithiana* (Smith's amanita or Lepidella), *Amanita proxima* (near neighbor amidella), and others.[88] The former contains toxins (eg, a 7 kDa basic cytotoxin) that damage the tubular epithelium, while the latter contains orellanines (eg, 3,3′-4,4′-tetrahydroxy-2,2′-bipyridyl-*N*,*N*′-dioxide from *C orellanus*) that induce delayed renal tubular necrosis, which leads to renal failure.

Most renal pathology associated with venom, plant, or fungal toxins is secondary. In snakebite, it may follow coagulopathy and bleeding, myolysis, hemolytic anemia, or a period of hypotension/shock. Venoms causing major hemolysis are associated with renal failure, as seen after massive multiple hymenopteran stings such as those inflicted by bees or vespid wasps, as well as after some snakebites. Recluse spider bites may also cause hemolysis as part of widespread tissue injury (viscerocutaneous loxoscelism), with associated multiple organ involvement, including renal failure.

The nature of the kidney injury depends on the toxin mechanism or that of the secondary effects. In snakebites, the injury is often due to acute tubular necrosis, from which full recovery after a period of hemodialysis is possible. However, cases of bilateral renal cortical necrosis are reported, where renal recovery occurs far less frequently.[35] Renal dysfunction secondary to hemolysis is similarly likely to be transient, but renal pathology following orellanine mushroom poisoning is permanent in some cases.

Treatment for possible impending nephrotoxicity is firstly preventative, by ensuring adequate cardiovascular function and hydration. Renal output must be carefully monitored, and hydration appropriately adjusted, sometimes with the addition of diuretics. For envenoming where antivenom is available, early neutralization of venom may help prevent or moderate renal damage, but in some snakebites a microangiopathic hemolytic anemia (with secondary renal damage) can occur despite early and adequate antivenom treatment. The biomedical basis of this phenomenon is not well characterized.

Once significant renal failure has developed, dialysis—preferably hemodialysis—is the key to management. In many developing countries renal dialysis facilities are uncommon and because of their unavailability, some envenomed patients who may have otherwise survived die from renal failure. However, some severe patients may be poorly responsive to dialysis and have fatal outcomes despite aggressive treatment.

Necrotoxins

Necrotoxins are major components in some venoms, such as those of the recluse spiders (family *Sicariidae*, *Loxosceles* spp) and the Iranian scorpion, *Hemiscorpius lepturus* (family *Hemiscorpiidae*; this species does not have a generally used common name, but the genus is sometimes collectively called the Asian thin-tailed scorpions); while cytotoxic and hemorrhagic toxins in some snake venoms may cause similarly direct injury and necrosis around the bite site. Although several species of *Hemiscorpius* are found in Iran, Pakistan, Yemen, and Iraq, *H lepturus* occurs only in Iran and is the only known medically important taxon in the genus. There are no available data regarding the possible medical significance of other species, and these should be treated as dangerous until proven otherwise.

Recluse spider (*Loxosceles* spp) venoms contain sphingomyelinase D that initiates neutrophil recruitment and thereby ultimately stimulates neutrophil-induced cell lysis at the bite site. This destructive neutrophil response has been linked to locally elevated levels of complement such as C5a. Interestingly, sphingomyelinase D from *Loxosceles* spp venom also activates target cellular matrix proteases that cleave the C5a receptor, thereby initiating a protective mechanism against the elevated levels of C5a.[89] The sphingomyelinases also alter the morphology of target cell membranes by transforming sphingomyelin into ceramide-1-phosphate. However, there are many gaps in the characterization of the precise pathophysiological mechanisms of some necrotoxins such as sphingomyelinase. Further elucidation of these mechanisms could provide information important to characterization of regulatory mechanisms governing local complement levels and autoimmune cellular responses in a variety of pathological states.

Clinically, local necrosis is often painful and obvious from an early stage, but recluse spider bites may be painless with few visible clinical manifestations in the first 12 to 24 hours, followed by the development of a classic "target" lesion (necrosing blue-black central skin, with surrounding pallor and an outer ring of erythematous reaction). This lesion can evolve over several days into epidermal necrosis that may be painful, and local blistering occasionally occurs. Necrosis following selected snakebites is usually painful and often accompanied by blistering, either centrally or at the margins of the evolving demarcated necrotic focus.

H lepturus is a particularly medically important species in Iran.[90] It is the only scorpion species known to commonly cause hemoglobinuric nephropathy and subsequent renal failure. In a series of *H lepturus* envenomings involving children younger than 10 years, Afzali and Pezeshki[91] reported that 8% had a

fatal outcome and 2.2% succumbed to renal failure. Stings from this species also cause local edema and hemorrhagic effects.

Treatment of toxin-induced necrosis is controversial. Early debridement of necrotic tissue may sometimes help, but for recluse spider bite it may actually increase the necrotic area. For this reason, current opinion discourages debridement, but delayed debridement may be necessary.[92] Some patients have been treated with hyperbaric oxygen and other modalities, but have not resulted in consistent benefit, and thus cannot be generally endorsed without further supporting evidence.[92] Secondary infection can occur and may either accelerate the necrosis, or even be the more important contributor to the necrotic process. Therefore, careful infection control is important, including appropriate antibiotics, as determined by anaerobic and aerobic culture, sensitivity determination, and regional trends in antibiotic resistance.

Other Toxins

There are many lesser toxins that are either of minor clinical importance in humans, poorly understood, or clinically significant but uncommonly to rarely encountered. For example, the giant tropical and desert centipedes, particularly of the genus *Scolopendra* (class Chilopoda, family *Scolopendridae*), are semi-fossorial and often nocturnal, and are not frequently encountered. These fast-moving active predators can inflict a painful sting with a pair of modified front legs often called maxillipedes, or forcipules (with the tarsungulum being the fang-like business end[93]) that are associated with pressurized venom glands (Figure 18-22). Although of generally minor importance, stings from several species have caused mild to moderate local effects (eg, pain [sometimes severe], edema, secondary infection and necrosis, pruritis), as well as uncommon systemic effects (eg, nausea, headache), some of which may also be due to autonomic responses to pain or anxiety. There are a few anecdotal reports of fatalities, and *Scolopendra subspinipes* has been viewed as a species of potentially greater medical importance in some rural Southeast Asian communities. An *S subspinipes* reportedly inflicted a fatal sting in a child in the Philippines.[94] These isolated reports require careful assessment and further documentation before assigning a defined hazard index for the species. However, the possible medical importance for giant centipedes may be underestimated in some regions, and rare deaths result from secondary complications of centipede stings (eg, necrotizing fasciitis).[95]

Several taxa and strains of the mold *Aspergillus* spp (family *Trichocomaceae*) produce difuranocoumarin derivatives, the aflatoxins, which are synthesized by a polyketide pathway. Interestingly, this fungal genus can be subjectively viewed as one that possibly contains the greatest contrasting mix of species because some provide respective benefits to humans (eg, utilization of *A niger* in the fermentation industry for citric acid synthesis[96]), yet others (eg, *A flavus*) are among the most medically hazardous, especially due to their toxins' carcinogenicity. The most studied aflatoxins (B1, B2, G1, G2) were named as a result of their blue or green fluorescence under ultraviolet light and their thin-layer chromatographic mobility properties.[96] These toxins are acutely toxic in sufficient doses and are also potent carcinogens.



Figure 18-22. The venom delivery apparatus of a scolopendrid centipede. The fang-like structures located on each side of the head are modified legs (maxillipedes or forcipules) that deliver venom into grasped prey or a potential predator. Thus, centipedes deliver venom through stinging, rather than via a bite.
Photograph: By Fritz Geller-Grim. Wikipedia Commons, public domain. https://commons.wikimedia.org/wiki/File:Scolopendra_fg02.JPG.

An accumulating body of interdisciplinary information suggests that interspecies variability in response to aflatoxins probably results from multifactorial influences including variability in the cytochrome P450 system that converts aflatoxins to the reactive, DNA adduct-forming, and protein-binding 8,9-epoxide forms.[97] Clinical signs of acute aflatoxicosis include hypolipidemia, hepatic steatosis, and necrosis.[98] Etzel[99] estimated the human LD_{50} (derived from murine data) of ingested aflatoxin B1 in liquid medium as 0.15 to 0.30 mg/kg. The longer term, carcinogenic consequences of aflatoxin exposure are a result of toxin binding to both

TABLE 18-4

PRINCIPAL TYPES OF MUSHROOM POISONING

Mushroom Clinical Group*	Principal Toxin(s)	Major Effect
Group 1 — Amatoxic mushrooms, notably *Amanita phalloides* (death cap) and related spp, as well as several other taxa (eg, *Lepiota* spp; Figure T1)	Amatoxins	Delayed-onset cytotoxicity via inhibition of RNA polymerases II that arrest transcription resulting in cellular (especially hepatocellular) destruction



Figure T1. *Lepiota* spp (*Agaricaceae*), Outer Banks, Nags Hags Head Woods, North Carolina. Some taxa of these gilled mushrooms (eg, *L helveola*) contain amatoxins and ingestion of several species including *L helveola* has caused fatalities. Photograph: By Jason Hollinger. Wikipedia Commons, public domain. https://commons.wikimedia.org/wiki/File:Lepiota_(4503849093).jpg.



Figure T2. Fly agaric (*Amanita muscaria*), Ulm, southern Germany. Typically not fatal, ingestion of these mushrooms can produce agitation, elation, disorientation, depersonalization, manic excitement, visual misperceptions (rather than true hallucinations), confusion; the syndrome can be mistaken for alcohol intoxication. There can be ataxia, incoordination, dizziness, mydriasis, myoclonus, muscle fasciculation/tremors, hyporeflexia, coma, and in severe poisoning, especially in children, convulsions. Ingestion of the congener, *A phalloides*, can be fatal, and there are a substantial number of well documented life-threatening and fatal cases.
Photograph: By Holger Krisp. Wikipedia Commons, public domain. https://commons.wikimedia.org/wiki/File:Amanita-muscaria-fliegenpilz-b.jpg.

Group 2 — Gyrometrin-containing mushrooms, notably *Gyromitra* spp	Gyrometrin (hydrolyzed to monomethyl hydrazine)	Delayed-onset cytotoxicity; cellular destruction
Group 3 – Coprine-containing mushrooms (various)	Coprine (*N*-5,1-hydroxycyclopropyl-L-glutamine)	Rapid autonomic effects, only in association with co-ingestion of alcohol (disulfarim-mimetic)
Group 4 – Muscarinic mushrooms, notably *Inocybe* spp, *Clitocybe* spp, *Mycena* spp	Muscarine (2,5-anhydro-1,4,6-trideoxy-6-(trimethylammonio)-D-ribo-hexitol)	Rapid-onset neuroexcitatory effects (parasympathetic stimulation; classically sweating, salivation, and lachrymation)
Group 5 — Ibotenic mushrooms, notably *Amanita muscaria* (Figure T2) and *Amanita pantherina*	Ibotenic acid, muscimol	Rapid onset of central nervous system effects, including agitation, elation, disorientation, mania, and visual misperceptions

(**Table 18-4** *continues*)

Table 18-4 continued

Group 6 — Hallucinogenic mushrooms, notably spp of *Psilocybe*, *Conocybe*, *Gymnopilus*, *Panaeolus*, and others	Psilocybin (4-phosphoryloxy-*N*,*N*-dimethyltryptamine; *note*: purchased hallucinogenic mushrooms may be laced with LSD and related substances)	Rapid onset of central nervous system effects including illusions (visual, auditory, or tactile), most commonly euphoric (but potentially sinister)
Group 7 — Gastrointestinal irritants, many species	Diverse array of toxins	Rapid onset (1–3 h) of gastrointestinal effects (vomiting, diarrhea, or abdominal pain)
Group 8 — Orellanine (nephrotoxic) mushrooms, notably *Cortinarius* spp	Orellanine (2,2′-bipyridine-3,3′,4,4′-tetrol-1,1′-dioxide)	Delayed-onset cytotoxicity; renal failure often occurs days after ingestion
Group 9 — Nephrotoxic mushrooms, notably *Amanita smithiana* and *A pseudoporphyria*	Aminohexadienoic acid	Delayed-onset cytotoxicity; renal failure with prerenal symptoms occurring 0.5–12 h later
Group 10 — Primary myolytic mushrooms, notably *Tricholoma equestre*	Unknown (*note*: these are normally edible mushrooms)	Delayed-onset myolysis due to unknown mechanism or association
Group 11 — Myolytic mushrooms, notably *Russula subnigricans*	Unknown	Rapid-onset myolysis
Group 12 — Pain-inducing mushrooms, notably *Clitocybe acromelalga*	Acromelic acid (several toxins; eg, acromelic acids A and B; all are heteroaromatics)	Rapid onset of rash, edema, and digit-tip pain (allodynia)

*The groupings listed here are based on the type of clinical presentation, rather than toxin type or mushroom species. Some groups may have many mushrooms, while others may have only a single species known to cause the specific clinical syndrome. From a clinical perspective, patients frequently ingest mushrooms of multiple species and potentially from multiple groups; thus, the clinical picture in any given patient may be a mixture of effects from a variety of mushroom groups. Clinical cognizance is especially important in relation to potentially lethal groups, particularly Group 1 amatoxic mushrooms, when coingested with other mushrooms causing gastrointestinal disturbance, as this may lead to a mixed clinical picture initially suggestive of poisoning by nonlethal Group 7 species.
Data source: (1) White J, Weinstein SA, De Haro L, et al. Mushroom poisoning: a proposed new clinical classification. *Clin Toxicol*. 2017 (under review).

DNA and RNA, which cause inhibition of replication, transcription, and protein synthesis; protein binding can also cause enzyme inactivation.[100]

Although it may seem unlikely that carcinogenic toxins might be used as biological warfare agents, significant evidence indicates that aflatoxins from *A flavus* and *A parasiticus* were a significant part of Iraqi bioweapons development during the 1980s[100] and were loaded into warheads and stockpiled.[101–103] Several possible reasons for their selection as potential offensive agents have been considered including obvious stimulation of fear and terror, and simple exploitation of favored or available resources.[97] However, consideration must be given to the possibility of intended infliction of longer term serious health effects on a given population (eg, as the initial Iraqi intent may have been directed against the Kurds in northern Iraq), a potential form of generational genocide. Thus, these toxins remain of concern on a relatively small scale because they are impractical as tactical weapons.[104]

Several filamentous fungi, particularly of the genus *Fusarium* (family *Nectriaceae*), produce tricothecenes such as T-2 and deoxynivalenol (vomitoxin), which are potent eukaryotic protein synthesis inhibitors and may disrupt initiation, elongation, and/or termination. There are several groups of these toxins including the nonmacrocyclic group (which contains an ester-ether bridge between C-4 and C-15 instead of a macrocyclic ester), which is further subclassified into structurally based groups, A and B.[96] T-2 is a potent immunotoxin, which binds to the 60S ribosomal subunit and subsequently inhibits elongation; it also inhibits protein synthesis induction.[105,106] Ingestion of T-2 toxin causes severe gastrointestinal distress including nausea, profuse diarrhea, vomiting, distention, and pain; those affected may also present with dizziness, chills, and other flu-like symptoms. T-2 toxins damage labile cells such as those in the gastrointestinal tract; they cause degeneration and necrosis of the lymphoid tissues and the surface and crypt epithelium of the gastrointestinal mucosa,[104] as well as induction of thymic lymphocytic apoptosis.[106] In humans T-2 toxin can induce apoptosis in megakaryocyte progenitors, and affected animals exhibit a notable loss of cell-mediated immunity.[104,107] Due to the acute effects caused by T-2 and its natural

TABLE 18-5

SELECTED PRINCIPAL TYPES OF PLANT POISONING

Plant Clinical Group	Principal Toxin(s)	Major Effects
CYTOTOXIC PLANTS		
Ricinus communis (castor bean, castor oil plant; Figure T3)	Ricin is a heterodimeric subunit protein that belongs to the ribosome inactivating protein family. One subunit (B chain) is a lectin and the other (A chain) is responsible for most of the toxicity.	Cytotoxic, inhibits protein synthesis by catalytic (via *N*-glycosidase) depurination of the 28S RNA, thereby arresting translation. The toxin can potentially cause lethal multiorgan failure, but most cases of simple ingestion (eg, raw ingestion of intact or slightly disrupted seeds) cause nonlethal gastrointestinal effects only.
Colchicum autumnale (autumn crocus, meadow saffron; Figure T4)	Colchicine (antimitotic; mitotic spindle poison)	Concentrates in polymorphonuclear leukocytes and inhibits microtubule assembly. Abdominal pain, vomiting, diarrhea, paralysis, convulsions, hypovolemic shock, respiratory failure, bone marrow failure.
Abrus precatorius (jequirty pea)	Abrin (also a ribosome inactivating protein)	Concentrates in polymorphonuclear leukocytes and inhibits microtubule assembly. Abdominal pain, vomiting, diarrhea, paralysis, convulsions, hypovolemic shock, respiratory failure, and bone marrow failure.

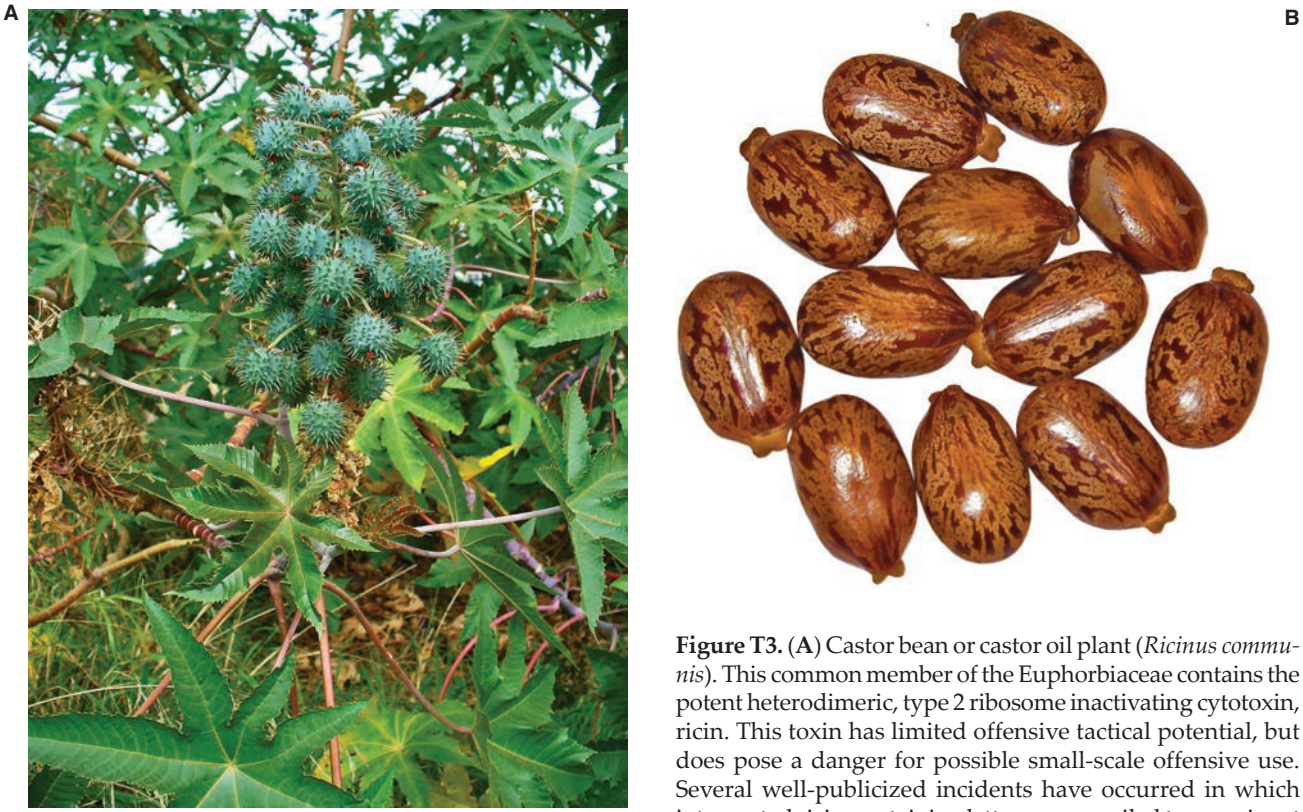


Figure T3. (**A**) Castor bean or castor oil plant (*Ricinus communis*). This common member of the Euphorbiaceae contains the potent heterodimeric, type 2 ribosome inactivating cytotoxin, ricin. This toxin has limited offensive tactical potential, but does pose a danger for possible small-scale offensive use. Several well-publicized incidents have occurred in which intercepted ricin-containing letters were mailed to prominent public figures, including the former Mayor of New York City, Michael Bloomberg, and President Barack Obama. This toxin has been used in several well-known perpetrated or attempted assassinations. (**B**) Castor bean (*Ricinus communis*) seeds. Photographs: By H Zell. Wikipedia Commons, public domain. (**A**) https://commons.wikimedia.org/wiki/Ricinus_communis#/media/File:Ricinus_communis_005.JPG. (**B**) https://commons.wikimedia.org/wiki/File:Ricinus_communis_008.JPG.

(**Table 18-5** *continues*)

Table 18-5 continued

Symphytum officinale (comfrey) and others	Pyrrolizidine alkaloids	Cyotoxic, toxic mechanisms uncertain, causes multiorgan damage, especially jaundice secondary to fibrosing hepatic venoocclusive disease.
Mentha pelugium (pennyroyal)	Pelugone	Cytotoxic, causing potentially fatal hepatotoxicity.



Figure T4. Autumn crocus or meadow saffron (*Colchicum autumnale*). This genus *Colchicaceae* contains more than 160 taxa. *Colchicum autumnale* is one of the species that contains the cytotoxic alkaloid, cochicine, an important pharmacotherapeutic especially used in the treatment of gout. Colchicine is a potent mitotic spindle poison, and has been suspected in several cases of intentional poisoning, but has no significant tactical potential.
Photograph: By Meneerke Bloem. Wikipedia Commons, public domain. https://commons.wikimedia.org/wiki/File:Colchicum_autumnale_clump_02.jpg.



Figure T5. Purple foxglove (*Digitalis purpurea*). The genus *Digitalis* contains approximately 20 species of perennials, shrubs and biennials. The leaves of some of these such as *D purpurea* contains the steroid, digoxin, the extract of which is used to produce digitalis and other related medications that are centrally important in the management of congestive heart failure and several dysrhythmias. Its mechanism of action targets the Na^+/K^+-ATPase pump on the cytoplasmic membrane of myocardiocytes (see Table 18-5), and acts as a positive inotrope. Its discovery is attributed to the Scottish physician, Dr William Withering, who reportedly obtained an early herbal mixture containing *Digitalis* spp from a gypsy, which led to his noting its effective therapeutic uses. Although it has no tactical potential, it has been used as a poison targeting individuals.
Photograph: Courtesy of Julian White.

(**Table 18-5** *continues*)

Table 18-5 continued

CYANOGENIC PLANTS		
Prunus spp (apricot, almond, peach, plum, apple, etc); hydrangea; cassava and others	Amygdaline	Metabolized to form hydrocyanic acid, causes metabolic failure, potentially lethal.
CARDIOTOXIC PLANTS		
Nerium oleander, Thevetia peruviana (oleanders; see Figure 18-20)	Cardiac glycosides (digitalis-like; oleandrin, nerium, thevetin, etc)	Cause digoxin-type cardiotoxicity by binding to and inactivating the Na^+/K^+-ATPase pump on the cytoplasmic membrane of myocardiocytes. As a result, the intracellular Na^+ concentration increases, and this affects the Na^+/Ca^{2+} exchange channels, thereby resulting in an increased intracellular Ca^{2+}. This causes a positive inotropic effect and also raises the resting membrane potential of the cell, leading to increasing rates of spontaneous cellular depolarization and myocardial automaticity. These effects can cause potentially lethal dysrhythmias, conduction anomalies, hyperkalemia, and death.
Carissa spectabilis (wintersweet)	Digoxin-like toxin	Cause digoxin-type cardiotoxicity by binding to and inactivating the Na^+/K^+-ATPase pump on the cytoplasmic membrane of myocardiocytes. As a result, the intracellular Na^+ concentration increases, and this affects the Na^+/Ca^{2+} exchange channels, thereby resulting in an increased intracellular Ca^{2+}. This causes a positive inotropic effect and also raises the resting membrane potential of the cell, leading to increasing rates of spontaneous cellular depolarization and myocardial automaticity. These effects can cause potentially lethal dysrhythmias, conduction anomalies, hyperkalemia, and death.
Digitalis purpurea, *D lantana* (foxglove) (Figure T5)	Digoxin	Cause digoxin-type cardiotoxicity by binding to and inactivating the Na^+/K^+-ATPase pump on the cytoplasmic membrane of myocardiocytes. As a result, the intracellular Na^+ concentration increases, and this affects the Na^+/Ca^{2+} exchange channels, thereby resulting in an increased intracellular Ca^{2+}. This causes a positive inotropic effect and also raises the resting membrane potential of the cell, leading to increasing rates of spontaneous cellular depolarization and myocardial automaticity. These effects can cause potentially lethal dysrhythmias, conduction anomalies, hyperkalemia, and death.
Convallaria majalis (lily of the valley)	Convallatoxin (cardiac glycoside)	As for other cardiac glycosides, but less potent.
Taxus breviffolia, T baccata (yew tree) (Figure T6)	Taxines (alkaloids derived from esterified diterpenes)	Cardiotoxins affecting cardiomyocytes by antagonizing sodium and calcium channels. These can potentially cause cardiac failure, but raw ingestions of plants are rarely lethal. The effects of isolated toxin ingestion are poorly documented, but probably carry a high risk of fatal outcome.
Veratrum spp (hellebore)	Veratridines (steroid alkaloids)	Act as agonists of the NaV and have neurotoxic and cardiotoxic effects. These toxins most commonly cause hypotension and bradycardia.

(**Table 18-5** *continues*)

Table 18-5 continued



Figure T6. Pacific Yew tree (*Taxus brevifolia*), Wenatchee Mountains, Wenatchee National Forest, Washington. The genus *Taxus* contains approximately seven species of monoecious or dioecious trees and shrubs. *Taxus* extracts have been long recognized as poisons, and the component alkaloids, the taxines, are potent cardiotoxins that antagonize cardiomyocyte ion channels (see Table 18-5). The yew tree also contains the diterpenes, the taxanes, which function as mitotic inhibitors. The very important chemotherapeutic agents, paclitaxel and docetaxel, are particularly used to treat a wide variety of solid neoplasms.
Photograph: By Walter Siegmund. Wikipedia Commons, public domain. https://commons.wikimedia.org/wiki/File:PacificYew_7790.jpg.



Figure T7. Angel's trumpet (*Brugsmansia* spp). The genus *Brugsmansia* consists of about seven Solanaceae taxa that feature large flowers with notably strong aroma. Ingestion of parts of these plants causes a classic anticholinergic syndrome, including delirium, hallucinations, tachycardia, blurred vision, dry mucosa, and seizures. Serious poisoning can result in death.
Photograph: Courtesy of Julian White.



Figure T8. Belladonna, deadly nightshade (*Atropa belladonna*). Another genus in the family *Solanaceae*, and the source for several very important tropane alkaloids used as pharmacotherapeutics: atropine, scopolamine, and hyoscyamine. The genus is named for Atropos, one of the three female deities who supervised fate, the Three Fates, and was the one who cut the thread of life. This is a worthy appellation, because these plants also can cause a potentially fatal classic anticholinergic syndrome as noted for Brugsmansia (see Figure T7 caption and Table 18-5).
Photograph: By Rüdiger Kratz. Wikipedia Commons, public domain. https://commons.wikimedia.org/wiki/File:Atropa-bella-donna_Staude_102_b.jpg.

(**Table 18-5** *continues*)

Table 18-5 continued

ANTICHOLINERGIC PLANTS		
Brugmansia spp (angel's trumpet) (Figure T7), *Datura* spp (jimsonweed), *Atropa belladonna* (deadly nightshade) (Figure T8), *Hyoscamus niger* (henbane), *Mandragora officinarum* (mandrake), and others	Anticholinergics (scopolamine, atropine, hyoscyamine, etc)	Classic anticholinergic toxidrome (delirium, hallucinations, pupillary dilatation, blurred vision, dry skin/mucosa, hyperthermia, flushed skin, tachycardia, hypertension, potentially urinary retention, coma, convulsions, and death).
NEUROTOXIC PLANTS		
Nicotiana spp (tobacco tree)	Nicotine (bicyclic alkaloid; structurally resembles acetylcholine, the ligand for the receptor to which it binds)	Binds with the nicotinic acetylcholine receptor and causes excitation with tremor, tachycardia, sweating, convulsions, gastrointestinal effects, then depressant effects including coma, paralysis, bradycardia, and cardiovascular collapse; potentially fatal.
Conium maculatum (poison hemlock)	Coniine (piperidine alkaloid)	Causes nicotine-like effects (see nicotine, above).
Aconitum spp (aconite)	Aconitine (aconitum alkaloid)	Acts as an agonist of tetrodotoxin-sensitive NaV; causes excitation, widespread paresthesia, muscle weakness, hypotension, cardiac arrhythmias, gastrointestinal effects (vomiting, diarrhea, abdominal pain), sweating, lacrimation, confusion, headache, and death.
Cicuta spp (water hemlock)	Cicutoxin (a C17-conjugated polyacetylene)	Noncompetitively antagonizes γ-aminobutyric acid (GABA) receptors thereby cause unregulated central nervous system neuronal depolarization. Delayed onset of oral mucosal pain (oral ingestion), abdominal pain, vomiting, coma, frothing at the mouth, convulsions, and death.
Laburnum anagyroides (*Laburnum*; *Faboideae*)	Cytisine (tricyclic quinolizidine alkaloid)	Acts as a partial agonist of nicotinic acetylcholine receptors containing specific combinations of the α4 and β2 subunits. Delayed-onset salivation, sweating, vomiting, delirium, excitation, convulsions, respiratory paralysis, and death.
Solanum spp (nightshades, bittersweet, Jerusalem cherry, potato; Solanaceae)	Solanine and related glycoalkaloids	Function as reversible inhibitors of human plasma cholinesterase, and may also be cytotoxic. Poisoning may include vomiting, diarrhea, dilated pupils, drowsiness, cholinesterase inhibition, respiratory failure, and death.
Cannabis sativa (marijuana)	Cannabinoids (tetrahydrocannabinol)	Depression or excitation, tremors, hallucinations, and aberrant behavior.
Cycads (number of taxa belonging primarily to *Cycadaceae* and *Zamiaceae*)	Cycasin (a nitrogen-containing methylazoglucoside)	In mammals, cycasin undergoes modification (cleavage) in vivo and forms methylazoxymethanol resulting in acute intoxication. It is probably teratogenic and is a tumor initiator in experimental rodents. It has been strongly implicated as a cause of Pacific parkinsonism dementia/amyotrophic lateral sclerosis complex.
NEPHROTOXIC PLANTS		
Rheum rhaponticum (rhubarb; *Polygonaceae*)	Oxalates	Soluble oxalates cause local irritation/corrosion and potential renal damage related to the excretion of the oxalate crystals.

(**Table 18-5** *continues*)

Table 18-5 continued

GASTROINTESTINAL IRRITANTS		
Juniperus sabina (juniper; *Cupresaceae*)	Diterpenics (communic acids; *J communis* also contains isocupressic acid, an abortificant)	Ingestion of oils, berries, and other parts of the plant can cause vomiting and diarrhea.
Wisteria spp (wisteria; *Fabaceae*)	Wisterin (glycoside most commonly reported from seeds and pods)	Reports suggest that ingestion causes gastroenteritis that may be severe, particularly in children.
Melia azedarach (white cedar; *Meliaceae*)	Limonoids (oxygenated, modified triterpenes) and other triterpenoids	Ingestion of the fruits can cause gastrointestinal effects, coma, and convulsions; potentially fatal in severe cases.
LOCAL IRRITANTS		
Rheum rhaponticum (rhubarb, *Polygonaceae*), *Dieffenbachia* spp (dumbcane, *Araceae*), *Zantedeschia aethiopica* (arum lily, *Araceae*), *Philodendron* spp (*Araceae*), and other oxalate-containing plants	Oxalate salts	Oxalate crystals released when plant (especially parts that contain high concentrations such as Rheum leaves) is chewed, causing local pain and edema; may cause dermal irritation in some individuals.
Euphorbia spp (poinsettia, candelabra cactus, etc, *Euphorbiaceae*)	Phorbol esters (tigliane diterpenes)	Contact with sap causes intense local irritation to mucosal membranes and the eye; some authors have reported potentially blinding effects. These compounds mimic the action of diacylglycerol; thereby may activate protein kinase C and function as tumor promoters.
Toxicodendron spp (poison ivy, poison sumac, and poison oak; *Anacardiaceae*), *Metopium* spp (poisonwood, *Anacardiaceae*)	Urushiols (3-substituted catechols)	Contact with the plant, including the intact leaves causes local skin irritation. Some species (eg, *T verniciflua*, *Melanorrhea usitata*) containing urushiol or related irritants are sometimes used in the preparation of furniture varnish and may present an occupational irritant hazard.

LSD: lysergic acid diethylamide; NaV: voltage-gated sodium channel
Data sources: (1) Brent J, Wallace KL, Burkhart KK, et al (eds). *Critical Care Toxicology: Diagnosis and Management of the Critically Poisoned Patient*. 1st ed. Philadelphia, PA: Elsevier-Mosby; 2005: 1,690 pp. (2) Riet-Correa F, Psister J, Schild AL, Wierenga TL (eds). *Poisoning by Plants, Mycotoxins and Related Toxins*. Oxfordshire, England: CABI; 2011: 660 pp. (3) Information synthesized from lectures presented by the authors.

occurrence in rice, it remains an agent that attracts concern about possible offensive use. However, as noted in regard to aflatoxins, such reasonable concern should be balanced with their impracticality for tactical applications.

The toxins in cytotoxic mushrooms, particularly the potent transcription inhibitors, the bycyclic heterogeneous octapeptide amatoxins, are clinically important because they regularly cause fatal poisoning after accidental ingestion. Cytotoxic mushrooms include the death cap (*Amanita phalloides*) and other gilled mushrooms that may closely resemble edible species such as some *Lepiota* spp (Table 18-4 and Figure T1). Amatoxin acts by inhibiting DNA-dependent RNA polymerase II, thereby stimulating hepatocellular acute death and apoptosis. Amanita poisoning is characterized by delayed—typically 8 to 12 hours—severe gastrointestinal effects, followed by apparent recovery; 2 to 4 days later, progressive onset of liver failure occurs, which can be fatal. Early recognition of the risk, timely charcoal decontamination when possible, and treatment with silibinin, penicillin G, or D-penicillamine may reduce the severity of liver damage, but after closure of the early treatment window, patients with major liver failure can only be managed with liver transplant. However, transplants can be either medically impractical because the patient is already too ill or a transplantable liver is unavailable.

The hallucinogens (eg, alkaloids such as mescaline, indole alkaloids such as psilocybin) and other nonlethal toxins found in some mushrooms (eg, *Psilocybe* spp, "magic mushrooms") or cactus (eg, the spineless cactus, *Lophophora williamsii*, peyote) cause characteristic clinical effects (see Table 18-4). Treatment varies with the toxin type, but is most commonly symptomatic and supportive. Some *Psilocybe* spp are sold for recreational purposes in Amsterdam, and thus are legally available in quantity.

There is a vast array of poisonous plants. A particularly well-studied example of a potent plant-derived cytotoxin is ricin, the seed-derived heterodimeric, type 2 ribosome-inactivation protein from *Ricinus communis* (castor bean or oil plant, Euphorbiaceae; Table 18-5 and Figures T3A, B)[108]. Ricin contains A and B polypeptide chains with molecular masses of 32 and 34 kDa, respectively, that are covalently linked via a single disulphide bond. The lectin-binding properties associated with the B chain aids entry into the target cell, which leads to endocytic vesicle internalization, thereby facilitating its retrograde transport through the Golgi and endoplasmic reticulum. It enters the cytosol, where the A-chain re-natures and then inactivates ribosomes.[107] The ribosomal inactivation is effective because one molecule of A chain can inactivate 1,777 ribosomes per minute,[109] and is enzymatically accomplished because the A chain is a highly active N-glycosidase responsible for selectively deadenylating the first adenine in a GAGA sequence in the α-sarcin/ricin loop of 28S rRNA.[107,110] Removal of this adenine prevents mammalian elongation factor-2 from binding to the ribosome,[111] thus blocking protein synthesis and activating apoptotic pathways.[112] Ricin poisoning can lead to fulminant multiorgan failure and death. Bozza et al (2013) list at least seven incidents involving potential terrorist actions or assassinations (planned or completed) involving ricin.[109] Therefore, although having limited offensive military potential, ricin still poses a significant danger for possible small-scale offensive use.

Other plant toxins could conceivably be used in a limited offensive manner like ricin. Selected principal types of plant poisoning are shown in Table 18-5.

CONCLUSIONS AND DIRECTIONS FOR RESEARCH

Only a relatively small minority of natural toxins (especially animal-derived) have been thoroughly characterized, and the clinical effects and management of this limited group are similarly well understood. Most venomous and poisonous organisms have yet to be subjected to any toxinologic research, so their possible risk to humans is unknown. Unfortunately, limited public and private funding is available for most toxinological investigations.

There is limited potential for offensive military applicability of venom toxins because of their physical and biochemical characteristics (eg, unpredictable stability of some purified toxins), as well as limitations on practical use per routes of administration, and even marked species-specificity of some toxins. The experimental lethal potencies of some venom toxins are notable and far greater than that of cyanide (eg, the presynaptic neurotoxin textilotoxin, from venom of the Australian Eastern brown snake [murine ip LD_{50} 1 μg/kg] is approximately 3,000-fold more potent than that for oral ingestion of sodium cyanide by nonfasted rats [3 mg/kg]).[113] However, bacterial toxins such as botulinum toxins a–g (murine LD_{50} 1–2 ng/kg; toxin d does not have affinity for human tissues)[114,115] or readily dispersible organophosphate nerve agents such as the cholinesterase inhibitor sarin (methylphosphonofluoridic acid L-methylethyl ester; acute toxicity [LCt_{50}] in resting humans estimated to occur with inhalation of 70 mg–min/m^3 aerosolized agent)[116] are far more concerning as offensive biological weapons. This concern is largely because of their toxicity and potential practical application per mass delivery or population exposure. Furthermore, although textilotoxin is the snake venom toxin with the highest experimental (murine) lethal potency, few patients envenomed by *Pseudonaja* spp whose venoms contain this toxin present with—or develop—paralytic features. In contrast, very low doses of either botulinum toxins or sarin produce severe neurotoxicity in humans with a high risk of lethal outcome.

However, some plant toxins (eg, ricin), as well as fungal toxins (eg, aflatoxins such as aflatoxin B1, and the trichothecenes T-2 toxin and deoxynivalenol), have either been offensively used in a small scale (in political assassinations), or have been suspected of being deployed against small populations (eg, suspected yellow rain in Southeast Asia and Afghanistan).[104] They remain a threat as a biological weapon, as does the possible small scale offensive application potential of tetrodotoxin.

Even for well-known causes of envenoming or poisoning, major gaps in knowledge exist, and optimized (or unambiguous) evidence-based treatment strategies have not been developed. Where antidotes such as antivenom are available, they may not be optimal in design and function, and no viable antivenoms can counter the effects of the venoms from many medically important venomous animals.

An important starting point for toxinology research is to understand the epidemiology of envenoming and poisoning so realistic risk profiles can be generated. Such data are often scant and fragmentary, or of questionable validity. Major methodological hurdles exist in collecting valid and useful epidemiologic data on toxin-induced diseases, not least because even the taxonomy of some of the fauna and flora of concern remains incomplete or uncertain. These problems are compounded by the tendency in developing nations—particularly in rural populations—for patients to be treated outside the health system, mostly by native healers, witch doctors, or shamans. Thus, even where government data on presentations for envenoming and poisoning are available, they may represent only a small minority of the actual percentage of the population affected by venomous or poisonous animals, fungi, and plants. Therefore, it is necessary to conduct detailed community-based surveys such as the Million Death Study of snakebite mortality in India,[117] in addition to garnering prospective hospital-based statistics.

Once important risk organisms are identified, a targeted toxin identification process is required if the toxins have not been previously elucidated. This process may require complex fractionation of the venom or poison to locate relevant toxins, including test systems to identify important potential activities. Sometimes a global "fishing" approach may be required, as used in the discovery of the huge diversity of action of cone snail toxins, where systematic injection of each individual toxin into test mice revealed a complex array of precisely acting toxins. This facilitated a detailed examination of individual toxins to understand how they exerted their often highly specific action in the central nervous system. Both in vitro and in vivo experimentation are usually required to understand the potential clinical effects of a given toxin and the mechanistic basis of its pathophysiological effects.

Recognizing the risks associated with a venomous or poisonous species and then identifying the medically important toxins opens the door to development of specific treatments. These treatments may be either pharmacologic antidotes to specific toxin actions, or antibody-based antidotes such as antiserums, developed to neutralize either specific toxins (eg, cardiotoxins such as oleandrin that are neutralized by anti-digoxin antibodies) or antivenoms developed against mixtures of toxins present in venoms. Efforts to standardize and optimize production of antivenoms have recently been discussed in detail in a World Health Organization publication, which is essential reading for those undertaking antivenom production.[118]

Biomedical research identifying animal and plant-derived toxins of medical importance and their biological activities should be elevated to a significantly greater level of priority. It is also essential that the evidence-based assessment of medical management of envenoming and other toxin-based diseases and emergencies should be recognized as a significant public health problem among a large proportion of the world's population.[119-121] The World Health Organization recently removed snakebite from its previous inclusion as an "other neglected tropical disease." Thus, the unknown and undoubtedly substantial, as well as underestimated, human cost of envenoming in many economically disadvantaged nations remains very much "neglected." The likelihood of military deployments in some of these regions adds to the importance of carefully assessing and addressing the risks of venom diseases and poisoning syndromes, and this is where the military importance of venomous animals and their venoms, as well as poisonous organisms and plants, are most relevant.

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Chapter 19

MARINE ALGAL TOXINS OF CONCERN AS INTENTIONAL CONTAMINANTS

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INTRODUCTION
Paralytic Shellfish Poisoning
Neurotoxic Shellfish Poisoning
Amnesic Shellfish Poisoning
Palytoxin

SUMMARY

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INTRODUCTION

Marine biotoxins are a problem of global distribution, estimated to cause more than 60,000 foodborne intoxications annually. In addition to human morbidity, some marine toxins may cause massive fish kills, such as those occurring during the Florida red tides. Others have been implicated in mass mortalities of birds and marine mammals. However, their presence in the environment is more often "silent," unless detected by monitoring programs or when contaminated foodstuffs are ingested.

The long-term environmental and public health effects of chronic exposure in humans have not been extensively studied, although questions are beginning to arise about whether chronic exposures to some marine toxins, such as okadaic acid, may increase the risk of cancer through their action as tumor promoters. Intoxication syndromes from ingestion of marine toxins have long been known, primarily associated with molluscan shellfish. These syndromes include paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), diarrhetic shellfish poisoning, amnesic shellfish poisoning (ASP), and azaspiracid poisoning. Another important marine intoxication is ciguatera fish poisoning, which is caused by ingesting contaminated finfish. Although for the most part, molluscan shellfish are the primary vectors to humans, filter feeding and other trophic transfers can result in occurrence in other seafood such as crustaceans and finfish.

With the exception of ASP, which is of diatom origin, the causative toxins all originate from marine dinoflagellates. More recently, palytoxin—a marine toxin originally described from zoanthid soft corals of the genus *Palythoa* in the Pacific Ocean, but now known to be produced by the dinoflagellate *Ostreopsis* spp—has become a potential human health problem in the Adriatic and Mediterranean seas. The toxin-producing algal species are a small fraction of the thousands of known phytoplankton species. However, under the proper environmental conditions, they can proliferate to high cell densities known as blooms. During these blooms, they may be ingested in large quantities by zooplankton, filter-feeding shellfish, and grazing or filter-feeding fishes. Through these intermediates, toxins can be vectored to humans who consume the seafood.

In general, marine algal toxins are not viewed as important biological warfare threat agents for several reasons. Marine toxins occur naturally at low concentrations in wild resources, and extraction of large quantities is difficult. Most are nonproteinaceous and, therefore, not amenable to simple cloning and expression in microbial vectors. Although some toxins can be harvested from laboratory cultures of the toxic organism, yields are insufficient to supply the large amounts required for deployment in traditional biological warfare aerosols or munitions.

Targeting food supplies as an act of biological terrorism is a more likely scenario. The toxins occur naturally in seafood products in concentrations sufficient to cause incapacitation or death. The contaminated foodstuffs appear fresh and wholesome, and cannot be differentiated from nontoxic material except by chemical analysis, which obviates the need for isolation of large quantities of pure toxins and subsequent adulteration of the food supply. In theory, the toxic seafood needs only to be harvested and then introduced into the food supply at the desired location. Regulatory testing, if any, is typically done only at the harvester and distributor levels. The natural occurrence of these toxins in seafood may provide cover for an act of intentional bioterrorism.

In some cases, harvesting toxic seafood is difficult. In the case of ciguatoxin, contaminated fish are typically a small percentage of the catch, and levels of toxin within toxic fish tissues are extremely low. In other cases, harvesting could be easy. The United States and other countries maintain monitoring programs at the state and local levels to ensure consumer safety. On the US Gulf Coast, concentrations of toxin-producing dinoflagellate *Karenia brevis* in the water column are closely monitored. When cell numbers increase to levels suggestive of an imminent bloom, harvesting of shellfish is officially halted. The shellfish are then monitored by chemical analysis or mouse bioassay until toxin concentrations in the edible tissues fall to safe levels, at which point harvesting is allowed to resume. During the period when shellfish are toxic, information is made available through the news media and regulatory agencies to discourage recreational harvesting, and anyone could conduct surreptitious harvesting during such a time.

Of the six marine toxin syndromes, three—ciguatera fish poisoning, diarrhetic shellfish poisoning, and azaspiracid poisoning—are unlikely to be significant bioterrorism threats. Diarrhetic shellfish and azaspiracid poisoning cause mild to moderate intoxications that are self-limiting and likely to be mistaken for common gastroenteritis or bacterial food poisoning; the syndromes are unlikely to cause the sorts of disruptions sought by terrorists. Ciguatera fish poisoning can present a more serious intoxication, but toxic fish are difficult to procure. Acquiring sufficient toxin to launch a food-related bioterrorist attack of any magnitude is nearly impossible.

The three marine algal toxin syndromes with bioterrorism potential and their causative toxins (Table 19-1) are described in the following sections. In addition, a brief description of palytoxin and its physiological effects is presented. Some of these effects are of greater concern for homeland security than others. Issues that may impact or limit their potential use as weapons of bioterror will be discussed, followed by clinical aspects and treatment.

Paralytic Shellfish Poisoning

Description of the Toxin

PSP results from exposure to a family of toxins called paralytic shellfish poisons, or saxitoxins. Saxitoxin (STX) was the first known member of this family, named for the giant butter clam, *Saxidoma giganteus*, from which it was first isolated.[1] Later it was learned that STX is the parent compound of more than 20 derivatives of varying potency produced by marine dinoflagellates of the genera *Alexandrium* (previously *Gonyaulax*), *Pyrodinium*, and *Gymnodinium*, as well as several species of freshwater cyanobacteria. In the 1990s, STX was isolated from bacterial species associated with dinoflagellate cells, suggesting the possibility of a bacterial origin in at least some dinoflagellates.[2] STX also occurs in other benthic marine organisms, such as octopi and crabs, from which the ultimate source of toxin is unknown but assumed to be the food web.[3]

In humans, the greatest risk is associated with consumption of filter-feeding mollusks such as clams, mussels, and scallops that ingest dinoflagellate cells during bloom conditions or resting cysts from the sediment. The original toxin profiles in the dinoflagellate cells may be metabolically altered by the shellfish. Ingestion by humans results in signs and symptoms characteristic of PSP. Approximately 2,000 cases occur annually across regions of North and South America, Central and South America, Europe, Japan, Australia, Southeast Asia, and India. PSP-related fatalities have been reported in South Africa, Canada, Chile, Guatemala, and Mexico. Because of this, numerous monitoring programs are now in place worldwide, which have minimized risks and drastically reduced fatalities.[4] The overall mortality rate has been estimated at 15%,[5] although mortality is highly dependent on the quality of medical care received. Etheridge provides a review of PSP toxins from a human health perspective.[4]

Mechanism of Action

STX and its derivatives elicit their toxic effects by interacting with the voltage-dependent sodium channels in electrically excitable cells of heart, muscle, and neural tissue. High-affinity binding to a specific binding site (denoted neurotoxin binding site 1) on sodium channels blocks ionic conductance across the membranes, thereby inhibiting nerve polarization. Although voltage-dependent sodium channels in many

TABLE 19-1

COMPARISON OF SELECTED MARINE ALGAL TOXINS

	Paralytic Shellfish Poisoning	Neurotoxic Shellfish Poisoning	Amnesic Shellfish Poisoning
Toxin	Gonyautoxins (saxitoxin)	Brevetoxins	Domoic acid
Source	Marine dinoflagellates	*Karenia brevis*	*Pseudo-nitzschia* multiseries
Mechanism of action	Binds to site 1 of voltage-dependent sodium channels, leading to inhibition of nerve polarization.	Binds to site 5 of voltage-dependent sodium channels and prevents channel inactivation.	Binds to kainate and AMPA subtypes of glutamate receptors in the central nervous system, leading to excitotoxic effects and cell death.
Clinical manifestations	Circumoral parasthesias that may rapidly progress to the extremities. May result in diplopia, dysarthria, and dysphagia. Progression may lead to paralysis of extremities and respiratory musculature.	Symptoms similar to paralytic shellfish poisoning, but usually milder. Nausea, diarrhea, and abdominal pain. Neurological symptoms include oral parasthesias, ataxia, myalgia, and fatigue.	Vomiting, diarrhea, and abdominal cramps, which may be followed by confusion, disorientation, and memory loss. Severe intoxications may result in seizures, coma, or death.

AMPA: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid

tissues are susceptible to these toxins, pharmacokinetic considerations make the peripheral nervous system the primary target in seafood intoxications.

Clinical Signs and Symptoms

Ingestion. Ingestion of PSP toxins results in a rapid onset (minutes to hours) complex of paresthesias, including a circumoral prickling, burning, or tingling sensation that rapidly progresses to the extremities. At low doses, these sensations may disappear in a matter of hours with no sequelae. At higher doses, numbness can spread to the trunk, and weakness, ataxia, hypertension, loss of coordination, and impaired speech may follow. Death has been known to occur in as little as 3 to 4 hours.[6]

A 20-year retrospective analysis of PSP documented by the Alaska Division of Public Health from 1973 to 1992 revealed 54 outbreaks involving 117 symptomatic patients.[7] The most common symptom in these outbreaks was paresthesia, and 73% of patients had at least one other neurological symptom. Other documented symptoms in descending order of occurrence included perioral numbness, perioral tingling, nausea, extremity numbness, extremity tingling, vomiting, weakness, ataxia, shortness of breath, dizziness, floating sensation, dry mouth, diplopia, dysarthria, diarrhea, dysphagia, and limb paralysis.[7]

Approximately 10 outbreak-associated PSP cases are reported to the Centers for Disease Control and Prevention each year. Thirteen cases of neurological illness associated with consumption of pufferfish containing STX caught near Titusville, Florida, occurred in 2002.[8] All 13 symptomatic patients reported tingling or numbness in the mouth or lips. Additionally, eight patients reported numbness or tingling of the face, 10 patients reported these symptoms in the arms, seven patients reported these symptoms in the legs, and one patient reported these symptoms in the fingertips. Six of the 13 patients experienced nausea, and four reported vomiting. Symptoms began between 30 minutes and 8 hours after ingestion, with a median of 2 hours. The illness lasted from 10 hours to 45 days, with a median of 24 hours. All of these cases resolved.

At lethal doses, paralysis of the respiratory musculature results in respiratory failure. Intoxication of a 65-year-old female in the Titusville case series is illustrative. The patient experienced perioral tingling within minutes of meal ingestion. Her symptoms worsened over the next 2 hours, and she experienced vomiting and chest pain. Emergency department evaluation noted mild tachycardia and hypertension. Over the next 4 hours, she developed an ascending paralysis, carbon dioxide retention, and a decrease in vital capacity to less than 20% predicted for her age, which led to intubation and mechanical ventilation. She regained her reflexes and voluntary movement within 24 hours and was extubated in 72 hours.[9]

Children appear to be more susceptible than adults. The lethal dose for small children may be as low as 25 μg of STX equivalents, whereas that for adults may be 5 to 10 mg of STX equivalents.[10] In adults, clinical symptoms probably occur upon ingestion of 1- to 3-mg equivalents. Because shellfish can contain up to 10 to 20 mg equivalents per 100 grams of meat, ingestion of only a few shellfish can cause serious illness or death.[10,11]

Fortunately, clearance of toxin from the body is rapid. In one series of PSP outbreaks in Alaska resulting from the ingestion of mussels, serum half-life was estimated at less than 10 hours. In these victims, respiratory failure and hypertension resolved in 4 to 10 hours, and toxin was no longer detectable in the urine 20 hours postingestion.[11] A paper describing a 2007 outbreak in Maine evaluated the clearance of individual PSP toxin congeners from the urine.[12] Half-lives were shorter for the sulfated derivatives GTX (gonyautoxin) 1/4 and GTX 2/3 (5–6 h) than for the parent toxins STX and neoSTX (16–20 h), suggesting that the toxin profile in the ingested shellfish may be an important factor in recovery time.[12]

Inhalation. In mice, STX is considerably more toxic by inhalation (LD_{50} of 2 μg/kg) or by intraperitoneal injection (LD_{50} of 10 μg/kg) than by oral administration (LD_{50} of 400 μg/kg).[13] Unlike PSP in humans, which is an oral intoxication and has a lag time to toxicity resulting from adsorption through the gastrointestinal tract, inhalation of STX can cause death in animals within minutes. At sublethal doses, symptoms in animals appear to parallel those of PSP, albeit with a more rapid onset reflective of rapid absorption through the pulmonary tissues.

Cause of Death

The cause of death in human cases of STX ingestion, as well as in experiments with animal models, is respiratory failure. Postmortem examination of STX victims reveals that the most notable effects are on the respiratory system, including pulmonary congestion and edema, without abnormalities of the heart, coronary arteries, or brain.[6,14] In vitro, STX does not directly affect the smooth muscle of airways or large blood vessels, but in vivo axonal blockade may lead to respiratory failure and hypotension.[15] Intoxication with large doses of STX may lead to metabolic acidosis, cardiac dysrhythmias, and cardiogenic shock, even with correction of ventilatory failure.[16] For patients that survive 24 hours, the prognosis is good, regardless of respiratory support.

Diagnosis

Clinicians should consider PSP patients who present with rapid onset of neurological symptoms that are sensory, cerebellar, and motor in nature and occur shortly after the consumption of seafood. Confirmatory diagnosis should rely on an analysis of body fluid samples, as well as an analysis of gastric contents or uneaten portions of recent meals. Urine, which is the primary mechanism of elimination, is used for analysis; the residence time of PSP toxins in the serum is short.[12] Animal studies and human intoxications both indicate toxins present in the urine for several days postingestion, although this time frame is dose dependent. Urine samples should be collected as soon as possible to ensure accurate analysis.

Postmortem examinations of fatally intoxicated humans have identified STX in gastric contents, body fluids (serum, urine, bile, and cerebrospinal fluid), and tissues (liver, kidney, lungs, stomach, spleen, heart, brain, adrenal glands, pancreas, and thyroid).[6,14] The largest concentrations of toxin existed in the gastric contents and urine.

Food or clinical samples can be evaluated by several methods. The traditional "gold standard" method is the mouse bioassay, which is an official method of the Association of Official Analytical Chemists International. High performance liquid chromatography, which is also an official method, can detect individual toxin congeners, but requires either precolumn or postcolumn derivatization of toxin mixtures for optimal detection.[12,17,18] Receptor binding assays based on either rat brain membranes[19] or purified STX binding proteins from frogs or snakes[20] measure total toxicity rather than individual toxin profiles. All of these have been used to detect PSP in urine and serum of intoxicated victims.[11] Immunoassays can detect individual toxins, but cross-reactivity among different congeners is highly variable; although useful in some situations, good correlation with analytical methods has so far been problematic. Rapid test kits are commercially available.

Medical Management

Treatment of STX intoxication consists exclusively of supportive care. Patients may benefit from gastric lavage if ingestion is recent. Patients need to be monitored closely for 24 hours, and if signs of respiratory compromise occur, aggressive respiratory management should be instituted. Intravenous fluids should be used judiciously to maintain urine output and blood pressure. Large doses of STX or intoxication in patients with underlying medical conditions may lead to cardiovascular abnormalities including hypotension, T-wave inversions, dysrhythmias, and cardiogenic shock. Sodium bicarbonate may be required for correction of metabolic acidosis. Vasopressor agents may be used to maintain blood pressure and perfusion of vital organs. Dobutamine may be the preferred agent; in experiments with high doses of STX given intravenously to cats, dobutamine improved recovery over dopamine.[16]

Research into specific treatments has examined heterologous antibody therapy and pharmacologic agents to overcome inhibition of the voltage-dependent sodium channel. However, at this time, no specific chemotherapeutic or immunotherapeutic agents exist for STX intoxication.

Because of its high potency and relative stability, STX must be considered a potential bioterrorist threat agent. Toxins are easily isolated from laboratory cultures, but production constraints would limit the scope of an aerosol attack. The more likely threat is through the food supply, with the vector being naturally contaminated fresh shellfish. Blooms of the causative organism occur annually along both the Atlantic and Pacific coasts of the United States and Canada, as well as elsewhere around the world, often in underdeveloped nations with poor screening programs. Toxins can easily reach lethal levels in filter-feeding shellfish. Depuration is slow enough that in some areas, such as George's Bank, some shellfisheries are permanently closed to harvesting. Threats to the water supply are minimal. Small-scale contamination (eg, of water coolers) is feasible, but large-scale contamination of reservoirs or even water towers is unlikely to be successful because of dilution effects coupled with the reduced potency of the oral route.

Neurotoxic Shellfish Poisoning

Description of the Toxin

NSP results from exposure to brevetoxins, a group of cyclic polyether toxins produced by the marine dinoflagellate *K brevis* (formerly *Ptychodiscus brevis* or *Gymnodinium breve*). Blooms of *K brevis*, with the associated discolored water and mass mortalities of inshore fish, have been described in the Gulf of Mexico since 1844.[21] As are paralytic shellfish poisons, brevetoxins are typically vectored to humans through shellfish; although in the case of NSP, the proximal agents are actually molluscan metabolites of the parent brevetoxins.[22] In addition to causing NSP, annual blooms of *K brevis* in the Gulf of Mexico can cause significant revenue losses in the tourism and seafood industries. Beachgoers can be especially affected because the unarmored dinoflagellates are easily broken up by rough

wave action, and the toxins become aerosolized into airborne water droplets, causing respiratory irritation and potentially severe bronchoconstriction in people with asthma.

Historically, NSP has been virtually nonexistent outside the Gulf of Mexico. However, an outbreak was reported in New Zealand in 1993. Blooms of another dinoflagellate, *Chattonella verruculosa,* occurred in Rehoboth Beach, Delaware, in 2000 and caused a series of localized fish kills.[23] Although no cases of NSP were reported, these events suggest a possible NSP range extension.

Mechanism of Action

Brevetoxins exert their physiological effects by binding with high affinity and specificity to neurotoxin receptor site 5 on the voltage-dependent sodium channel.[24] Unlike STX, which inhibits the sodium channel by binding to site 1, binding of brevetoxins to site 5 prevents channel inactivation. This shifting of the voltage-dependence of channel activation leads to channel opening at lower membrane potentials[25] and inappropriate ionic flux. Clinical effects are typically more centrally mediated than peripherally mediated. Brevetoxins can cross the blood–brain barrier, and it hypothetically leads to injury and death of cerebellar neurons by stimulation of glutamate and aspartate release, activation of the *N*-methyl-D-aspartate (NMDA) receptor, and excitotoxic cell death.[26] A detailed review of the molecular pharmacology and toxicokinetics of brevetoxin can be found in Poli's *Recent Advances in Marine Biotechnology. Vol 7: Seafood Safety and Human Health*.[27]

Clinical Signs and Symptoms

Ingestion. Symptoms of NSP are similar to that of PSP, but are usually milder. Manifesting within hours after ingestion of contaminated seafood, symptoms include nausea, diarrhea, and abdominal pain. Typical neurological symptoms are oral paresthesia, ataxia, myalgia, and fatigue. In more severe cases, tachycardia, seizures, loss of consciousness, and respiratory failure can occur. During a 1987 outbreak, 48 cases of NSP occurred in the United States. Acute symptoms documented in the outbreak included gastrointestinal (23% of cases) and neurological (39% of cases) symptoms. Symptoms occurred quickly, with a median of 3 hours to onset, and lasted up to 72 hours. Most of the victims (94%) experienced multiple symptoms, and 71% reported more than one neurological symptom.[28]

Although a fatal case of NSP has never been reported, children may be more susceptible, and a fatal dose must be considered a possibility.[22] The toxic dose of brevetoxins in humans has not been established. However, important information has been gleaned from a clinical outbreak. A father and two small children became ill after ingesting shellfish harvested in Sarasota Bay, Florida, in 1996. Both children were hospitalized with severe symptoms, including seizures. Brevetoxin metabolites were detected in urine collected 3 hours postingestion. With supportive care, symptoms resolved in 48 to 72 hours, and no brevetoxin was detectable in the urine 4 days later.[22] Mass chromatography of serum samples taken immediately after the family checked into the hospital demonstrated ion masses suggestive of brevetoxin metabolites, although these compounds were never isolated. The amount of toxin ingested was not determined, although the father, who had milder symptoms and was released from the hospital after treatment, reported eating "several" shellfish. The number eaten by the children (ages 2 and 3) was unknown.

The toxicity of brevetoxins in mice is well established. LD_{50} values range from 100 to 200 μg/kg after intravenous or intraperitoneal administration for PbTx-2 and PbTx-3, the two most common congeners. Oral toxicity is lower: 500 and 6,600 μg/kg for PbTx-3 and PbTx-2, respectively.[29] Animal models indicate brevetoxin is excreted primarily in the bile, although urinary elimination is also significant. Toxin elimination is largely complete after 72 hours, although residues may remain in lipid-rich tissues for extended periods.[30]

Inhalation. Respiratory exposure may occur with brevetoxins associated with harmful algal blooms or "red tides." As the bloom progresses, the toxins are excreted and released by disruption of the dinoflagellate cells. Bubble-mediated transport of these toxins leads to accumulation on the sea surface; the toxins are released into the air by the bursting bubbles. The toxins are then incorporated into the marine aerosol by on-shore winds and breaking surf, leading to respiratory symptoms in humans and other animals. Sea foam may also serve as a source of toxin and result in symptoms if it is ingested or inhaled. During harmful algal blooms, the on-shore concentration of aerosolized toxins varies along beach locations by wind speed and direction, surf conditions, and exposure locations on the beach. Concentrations of the toxin are highest near the surf zone.[31]

Systemic toxicity from inhalation is a possibility. Distribution studies of intratracheal instillation of brevetoxin in rats have shown that the toxin is rapidly cleared from the lung, and more than 80% is distributed throughout the body. Twenty percent of the initial toxin concentration was present in several organs for 7 days.[32]

Diagnosis

Brevetoxin intoxication should be suspected clinically when patients present with gastrointestinal symptoms and neurological symptoms occurring shortly after ingesting shellfish. Although these symptoms may be similar to those of STX intoxication, they do not progress to paralysis. Epidemiological evaluation of cases may identify additional cases during an outbreak and allow for public health measures, including surveillance, to be put into place.

Human cases are typically self-limiting, with improvement in 1 to 3 days, but symptoms may be more severe in young persons, elderly persons, or those with underlying medical conditions. Evaluation of biological samples should include urine as well as any uneaten shellfish from the meal. Toxins in clinical samples can be detected by liquid chromatography mass spectrometry receptor-binding assays, or immunoassay. Because metabolic conversion of parent toxins occurs in shellfish and the metabolites are apparently less active at the sodium channel, it appears that immunoassays are better screening tools. However, secondary metabolism in humans has yet to be fully investigated.

Medical Management

No specific therapy exists for NSP. If the ingestion is recent, treatment may include removal of unabsorbed material from the gastrointestinal tract or binding of residual unabsorbed toxin with activated charcoal. Supportive care, consisting of intravenous fluids, is the mainstay of therapy. Although brevetoxin has not been implicated in human fatalities, symptoms of NSP may overlap with symptoms of STX and thus warrant observation for developing paralysis and respiratory failure. Aggressive respiratory management may be required in severe cases.

Pulmonary symptoms resulting from inhalation of marine aerosols typically resolve upon removal from the environment, but may require treatment for reactive airway disease, including nebulized albuterol and anticholinergics to reverse bronchoconstriction. Mast cell release of histamine may be countered with the use of antihistamines. Mast cell stabilizers, such as cromolyn, may be used prophylactically in susceptible persons exposed to marine aerosols during red tide events.

No antitoxins for NSP are available. However, experiments with an anti-brevetoxin immunoglobulin G showed that treatment before exposure blocked nearly all neurological symptoms.[33] Additional research into pharmacologic agents should be pursued. Two brevetoxin derivatives that function as brevetoxin antagonists but do not exhibit pharmacologic properties have been identified. Other agents that compete with brevetoxin binding for the sodium channel include gambierol, gambieric acid, and brevenal.[34,35] Future research with these agents may assist in developing adequate therapeutics.

Brevetoxins are likely to have only moderate potential as agents of bioterror. Although unlikely to cause mortality in adults, oral intoxication can be severe and require hospitalization. Disruption of a local event, inundation of medical facilities by the "worried well," and societal overreaction possibly leading to economic disruption of local industry are the most likely repercussions. *K brevis* is easily cultured and produces toxins well in culture. Unpublished animal experiments suggest brevetoxins may be 10-fold to 100-fold more potent by aerosol—versus oral—exposure. Thus, small-scale aerosol attacks are technically feasible, although isolation and dissemination of toxins would be difficult for nonexperts.

Amnesic Shellfish Poisoning

Description of the Toxin

ASP was defined after an outbreak of mussel poisoning in Prince Edward Island, Canada, in 1987. More than 100 people became ill with an odd cluster of symptoms, and three died. Canadian researchers quickly isolated the causative agent and identified it as domoic acid.[36] Domoic acid, which was previously known as a compound tested and rejected as a potential insecticide, is a common ingredient in Japanese rural folk medicine. Domoic acid was originally isolated from the marine red algae *Chondria* spp, and researchers were surprised to discover that the diatom *Pseudo-nitzschia pungens f multiseries* (now *Pseudo-nitzschia multiseries*) was the causative organism. ASP remains the first and only known seafood toxin produced by a diatom.

Since the 1987 outbreak, several toxic species of diatoms have been found around the world and are now the subject of many regional monitoring programs. Domoic acid is seasonally widespread along the US Pacific Coast and the Gulf of Mexico. It has also been found in New Zealand, Mexico, Denmark, Spain, Portugal, Scotland, Japan, and Korea. Although amounts of domoic acid in shellfish occasionally reach levels sufficient to stimulate harvesting bans, no further human cases have been reported, reflecting the efficacy of monitoring programs. However, the toxicity of domoic acid remains evident in biotic events.

In 1991, numerous cormorants and pelicans died after feeding on anchovies (a filter-feeding fish) during a bloom of *Pseudo-nitzschia australis* in Monterey Bay, California. High levels of domoic acid were detected in the gut contents of the anchovies. Later that year, after

the bloom moved northward along the coast, razor clams and Dungeness crabs became toxic off the Washington and Oregon coasts. Several cases of human intoxication apparently followed ingestion of razor clams, although a definitive link was not found.[37] More than 400 sea lions died and numerous others became ill in 1998 after ingesting anchovies feeding in a bloom of *P australis*, again in Monterey Bay.[38] Domoic acid was detected in both the anchovies and feces from the sea lions.[39] These events suggest that periodic blooms of domoic acid–producing *Pseudo-nitzschia* on the western coast of the United States may cause significant toxicity in seafood items.

Mechanism of Action

Domoic acid is a neuroexcitatory amino acid structurally related to kainic acid. As such, it binds to the kainate and AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) subtypes of the glutamate receptor in the central nervous system, which subsequently elicits nonsensitizing or very slowly sensitizing currents,[40] causes a protracted influx of cations into the neurons, and stimulates a variety of intracellular events leading to cell death.[41] This effect may be potentiated by synergism with the excitotoxic effects from high glutamate and aspartate levels found naturally in mussel tissue.[42] The kainate and AMPA receptors are present in high densities in the hippocampus, a portion of the brain associated with learning and memory processing. Mice injected with domoic acid develop working memory deficits.[43] Neuropathological studies of four human fatalities revealed neuronal necrosis or loss with astrocytosis, mainly affecting the hippocampus and the amygdaloid nucleus.[44] Developing and aging brains show higher susceptibility to domoic acid, and thus define susceptible subpopulations.

Clinical Signs and Symptoms

Ingestion. The 1987 Prince Edward Island outbreak provided information on the clinical effects of domoic acid ingestion in humans.[45] The outbreak occurred during November and December, with 250 reports of illness related to mussel consumption (107 of these reports met the classic case definition). All but seven of the patients reported gastrointestinal symptoms ranging from mild abdominal discomfort to severe emesis requiring intravenous hydration. Forty-three percent of patients reported headache, frequently characterized as incapacitating, and 25% reported memory loss, primarily affecting short-term memory.

At higher doses, confusion, disorientation, and memory loss can occur. Severe intoxications can produce seizures, coma, and death. Nineteen of the patients required hospitalization for between 4 and 101 days, with a median hospital stay of 37.5 days. Twelve patients required care in an intensive care unit. The intensive care patients displayed severe neurological dysfunction, including coma, mutism, seizures, and purposeless chewing and facial grimacing.[45] Severe neurological manifestations, which were more common in elderly persons, included confusion, disorientation, altered states of arousal ranging from agitation to somnolence or coma, anterograde memory disorder, seizures, and myoclonus. Although mean verbal and performance IQ scores were average and language tests did not reveal abnormalities, severe memory deficits included difficulty with initial learning of verbal and visuospatial material, with extremely poor recall. Some of the more severely affected patients also had retrograde amnesia that extended to several years before ingestion of the contaminated mussels.[44] Nine of the intensive care patients required intubation for airway control resulting from profuse secretions, and seven of them suffered unstable blood pressures or cardiac dysrhythmias. Three patients died during their hospitalization.[45]

Symptoms of intoxication occur after a latency period of a few hours. In mild cases, the gastrointestinal symptoms of vomiting, diarrhea, and abdominal cramps occurred within 24 hours. The time from ingestion of the mussels to symptom onset ranged from 15 minutes to 38 hours, with a median of 5.5 hours.[45] In a study of 14 patients who developed severe neurological manifestations, 13 developed gastrointestinal symptoms between 1 and 10 hours after ingestion, and all of the patients became confused and disoriented 1.5 to 48 hours postingestion. Maximal neurological deficits were seen 4 hours after mussel ingestion in the least affected patients and up to 72 hours postingestion in those patients who became unresponsive.[44] All the patients who developed severe neurological symptoms were older than 65 or had preexisting medical conditions such as diabetes or renal failure that altered their renal clearance.

Inhalation. No natural cases of domoic acid inhalation exist, and no experimental models have evaluated an aerosol exposure to this toxin. It may be assumed that the toxin would be absorbed through the pulmonary tissues leading to systemic symptoms comparable to other exposure routes, although no data confirm this theory.

Diagnosis

Diagnosis should be suspected by the clinical presentation after ingestion of filter feeding seafood. Patients may have mild symptoms that resolve spontaneously

or may present with more severe signs of neurotoxicity, including confusion, altered mental status, or seizures. Symptomatic patients typically are older than 65 or have underlying medical conditions that affect renal clearance.[45] Initial evaluation of these patients should include standard protocols for patients with altered mental status, including toxicological screens to rule out more common intoxicants, especially illicit substances. Other diagnostic tests can be used to rule out other clinical causes of the symptoms including imaging with computed tomography scans, which does not show abnormality related to domoic acid intoxication, and monitoring of brain activity with electroencephalography. Of the 12 patients that were admitted to the intensive care unit during the 1987 outbreak, electroencephalograms showed that nine had generalized slow-wave activity and two had localized epileptogenic activity.[45] Positron emission tomography scanning of four patients with varying degrees of illness revealed a correlation between glucose metabolism in the hippocampus and amygdala with memory scores.[44]

Based primarily on levels measured in Canadian shellfish after the 1987 outbreak, mild symptoms in humans may appear after ingestion of approximately 1 mg/kg of domoic acid, and severe symptoms may follow ingestion of 2 to 4 mg/kg. The official regulatory testing method uses analytical high performance liquid chromatography, although both immunological methods and a simple, inexpensive thin-layer chromatography method are available.[46–48] No evidence of domoic acid metabolism by rodents or primates exists, as shown by recovery in an unchanged form from the urine or feces.[49] Samples to be included for definitive testing include serum, feces, urine, and any uneaten portions of the suspected meal.

Medical Management

Treatment for intoxication with domoic acid is supportive care. For patients who present early after ingesting the meal, gastric lavage or cathartics may decrease toxin amounts absorbed systemically. A key issue with this intoxication is the maintenance of renal clearance; hydration or other measures may also be required. Additionally, severe intoxications may cause alterations in hemodynamic functions, requiring pharmacologic interventions to maintain perfusion. In the 1987 outbreak, some severely intoxicated patients developed substantial respiratory secretions requiring intubation. Patients should be monitored for seizure activity that may require anticonvulsants. Studies in mice have shown that sodium valproate, nimodipine, and pyridoxine suppress domoic acid-induced spike and wave activity on an electroencephalogram.[50]

No specific therapy exists for domoic acid intoxications. Research has revealed that competitive and noncompetitive NMDA receptor antagonists reduce the excitable amino acid cascade that leads to brain lesions.[51] Additionally, NMDA receptor antagonists have also been shown to antagonize domoic acid toxicity.[51]

Domoic acid should be considered a legitimate—if moderate—bioterrorist threat agent. Toxic shellfish are available, and ingestion elicits symptoms that can be life threatening. Although mass casualties are not likely, mortality can occur, and the frightening nature of the symptoms in survivors may cause the disruption sought by an aggressor.

Palytoxin

Description of the Toxin

Palytoxins are a group of complex marine natural products originally isolated from zoanthid soft corals of the genus *Palythoa*. The structure of the first palytoxin was elucidated in 1981[52,53] when first described by Moore and Scheuer in 1971.[54] Palytoxins are complex hemiketals with molecular weights of approximately 2,600 to 2,700 g/mol, and containing cyclic ethers, 64 chiral centers, and multiple hydroxyl groups. The original palytoxin from *Palythoa toxica* contains a continuous chain of 115 carbon atoms, making it unique among known natural products.[55] Since that discovery, a family of palytoxins has emerged, including congeners from *Palythoa tuberculosa* and *Palythoa margaritae* in Japan, and *Palythoa caribaeorum* from Puerto Rico. In addition, a palytoxin-like compound was isolated from the sea anemone *Radianthus macrodactylus* from the Seychelles,[56] and two analogs have been isolated from the red alga *Chondria armata*, which also produces domoic acid.[57] More recently, dinoflagellates of the genus *Ostreopsis* have been shown to produce a variety of palytoxin analogs, including ostreocins and ovatoxins (from *Ostreopsis ovata* and *Ostreopsis siamensis*) and macarenotoxins from *Ostreopsis mascarenensis*.[55] Finally, both palytoxin and one of the newly described palytoxin analogs, 42-hydroxy-palytoxin, have been identified in a marine cyanobacterium of the genus *Trichodesmium* in New Caledonia, raising the possibility of additional food–web sources.[58]

In this chapter, all congeners, including ovatoxins-a–f, ostreocins, and palytoxins are collectively referred to as "palytoxins." In many of the references cited, the compounds referred to as "palytoxin" were not highly purified, or only compared to a chromatographically derived palytoxin standard. It is very likely that many

were actually mixtures of palytoxin analogs, which have only recently been described and are difficult to separate.

Palytoxins are some of the most potent nonproteinaceous toxins known. Intravenous LD_{50} values (in μg/kg) range from 0.025 (rabbits), 0.033 (dogs), 0.078 (monkeys), 0.089 (rats), and 0.11 (guinea pigs). Of the animal models tested, mice are the least susceptible at 0.15 μg /kg to 0.45 μg /kg, depending on the investigator and the origin of the toxin.[59,60] Potency is somewhat less by other routes. The lowest potency seems to be by the oral route, with LD_{50} values for palytoxin and ostreocin-D in the range of 500 μg/kg to 1,000 μg/kg in mice.[61–64]

In spite of the rather unimpressive oral toxicity values in mice, fatal human intoxications from palytoxins have been reported since the 1700s, primarily from grazing or filter feeding species of fish in the Atlantic and Pacific oceans and the Caribbean Sea.[65] Palytoxins are now thought to be the causative agent in the often fatal syndrome formerly known as clupeotoxism, which results from the ingestion of filter feeding fish of the family *Clupeidae* (herrings and sardines) and *Engraulidae* (anchovies) among others.[66] They have also been implicated in several fatal cases of poisoning from various species of xanthid crabs in the Philippines.[66] Palytoxins are known to bioaccumulate in important seafood species such as mussels, sea urchins, and cephalopods (octopus and squid), suggesting a real possibility of human intoxication from the ingestion of seafood.[67,68] Deeds and Schwartz[55] provide an excellent review of human intoxication from seafood.

More recently, palytoxins have begun to cause problems in the Mediterranean and Adriatic seas, probably as a result of the introduction of *O ovata* into these waters. Several instances of respiratory symptoms associated with blooms of *Ostreopsis* were reported in the early years of the 21st century along the coasts of Italy, Spain, and France (reviewed in Del Favero et al[69]). The most striking of these occurred in 2005, when more than 200 beachgoers near Genoa, Italy, experienced respiratory symptoms of bronchoconstriction, dyspnea, cough, and rhinorrhea. Of these 200, 20 beachgoers experienced symptoms severe enough to warrant extended hospitalization and intensive care. These symptoms occurred during a bloom of *O ovata* near the coast.[70,71] Symptoms peaked during the peak of the bloom and dissipated with the bloom. Cell samples of *O ovata* collected during the bloom tested positive for palytoxins by high performance liquid chromatography and mass spectrometry.[72] Similar testing from subsequent events has resulted in the identification of new palytoxin analogs, including ovatoxin-a, which may predominate in these blooms.[73] Blooms of *O ovata* now regularly occur in the Mediterranean and Adriatic seas,[69] raising important questions regarding public health, seafood safety, and the potential for the accumulation of large amounts of toxins through dinoflagellate culture. More importantly, the intoxication of beachgoers during periods of little wind from dinoflagellate blooms in the water column suggests high aerosol potency. Thus, aerosol toxicology studies of palytoxin are clearly warranted.

Mechanism of Action

The well-accepted, oft-cited mechanism of action of palytoxin is through interference of the Na^+/K^+-ATPase ionic pump, converting it to a nonspecific ion pore and altering the internal ionic concentration of susceptible cells.[60,61] Binding of palytoxin to the pump causes an immediate influx of Na^+, which triggers an influx of K^+ and Ca^{++}. This interference with intracellular ionic homeostasis, especially the increase in intracellular Ca^{++}, entrains a variety of toxic cell responses. Although inhibition of many of the toxic effects by ouabain supports this as the primary source of the pathophysiology of palytoxins, significant evidence from both in vivo and in vitro models indicate ancillary mechanisms as well (see the review by Munday[60]). Tubaro et al[62] provides a review of the in vitro and in vivo biological effects of palytoxin.

Clinical Signs and Symptoms

Ingestion. Manifestations of palytoxin intoxication by ingestion typically involve malaise, nausea, vomiting and diarrhea, a bitter or metallic taste in the mouth, myalgia and cramps, numbness or tingling in the extremities, bradycardia, and dyspnea. Renal failure has occurred in severe cases, probably secondary to the myoglobinemia associated with skeletal muscle damage. Human oral intoxications by palytoxins are reviewed in Yasumoto and Murata,[57] Deeds and Schwartz,[55] and Tubaro et al.[74] However, the example of a fatal intoxication occurring after the ingestion of a crab, *Demania reynaudii*, in the Philippines is illustrative.

In November 1984, at around noon, a man cooked a crab caught in a net off Tanjay Town in the province of Negros Oriental, Philippines.[66] Within minutes of partially ingesting the crab, he experienced dizziness, fatigue, nausea, and a metallic taste in his mouth. Later he experienced diarrhea. When a dog died that had eaten the remainder of his crab, the man requested transport to a local hospital. During the trip, he experienced fatigue, numbness in the extremities, restlessness, and vomiting. Upon admission at about

5:45, he complained of restlessness, muscle cramps, and vomiting. He died at 3:14 the next morning, approximately 15 hours postingestion.

Clinical records revealed alternating periods of normal heart rate and severe bradycardia (30 beats/min), rapid and shallow respiration, cyanosis around the mouth and in the hands, and renal failure (anuria).[66] Administration of atropine, diphenhydramine, pethidine, and adrenaline was ineffective. The causative agent was determined to be palytoxin based on the dose/survival time relationship in the mouse bioassay and the chromatographic characteristics of the extracted toxin, both of which were identical to those of a palytoxin standard.

Inhalation. Although *O siamensis* is well known around Japan, Australia, New Zealand, and the Mediterranean, *O ovata* has only recently become established in the Mediterranean/Adriatic. The first blooms occurred in 2003, and now have become regular events. Blooms associated with respiratory effects in humans occurred in 2003 and 2004 in Italy, and they have been reported in Spain, France, Croatia, Tunisia, Greece, and Algeria since that time.[75]

The most complete description of aerosol exposure to palytoxin is a Genoa event in 2005. In late July, during a period of warm weather with little wind and calm seas, a large bloom of *O ovata* occurred along the coastline. Concomitantly, a total of 209 beachgoers were afflicted with a complex of symptoms that included fever (64%), sore throat (50%), cough (40%), dyspnea (39%), headache (32%), nausea (24%), rhinorrhea (21%), lacrimation (16%), vomiting (10%), and dermatitis (5%).[71] Although no deaths occurred, 20 people were hospitalized, some for as long as 3 days. Mean onset of symptoms was 4.5 hours (range 30 min–23 h). Laboratory analyses were available for 82 patients, including all those hospitalized. Of these, 46% had leukocytosis (mean white cell count was 13,900/mm^2) and 40% had neutrophilia (mean 82%); transaminases, gamma-glutamyl transpeptidase, creatinine, and sedimentation rate values were normal. Chest X-rays and electrocardiogram values were normal.

Dermal. Dermal exposure to palytoxins can occur with exposure to water containing blooms of *Ostreopsis*, although this seems to be a minor hazard. For example, in the Genoa bloom event of 2005, only 5% of patients reported a mild dermatitis.[71] However, in describing bloom occurrences along the French Mediterranean coast, Tichadou et al reported skin irritation as the most common sign in people exposed to cells in the water column.[75] At low cell numbers, erythema resolved rapidly after exposure. At higher cell numbers, clinical findings included pruritis of exposed skin, conjunctivitis, rhinorrhea, and oral irritation.

Exposure Through Home Aquaria. A more serious dermal issue can occur through the home aquarium trade. Colonies of zoanthid corals are popular aquarium decorations. They readily propagate and provide a colorful marine backdrop to other species. Home aquarists are usually cognizant of the potential toxicity of zoanthid corals and handle them with gloves when cleaning aquaria or dividing colonies. Occasionally, however, intoxications occur. In one case, a 25-year-old woman handled a zoanthid coral without gloves in her home aquarium.[76] She noted a metallic taste in her mouth within minutes, followed by perioral paresthesias and hives on her torso and extremities. The next day she noted edema of the upper lip without airway compromise. By the second day, the paresthesia had resolved, but she experienced increasing edema, erythema, and pruritis in both hands. In addition, a bilateral urticarial rash was noted on her upper arms, thighs, abdomen, upper chest, and back. Symptoms improved after treatment with intravenous diphenhydramine, methylprednisolone, and lorazepam.

In another instance of aquarium dermal intoxication, a 32-year-old man was admitted to the emergency department 20 hours after cutting three fingers on his right hand on a zoanthid colony while cleaning his aquarium.[77] Within 2 hours, he exhibited shivering, myalgia, and general weakness of the extremities. After 16 hours, he collapsed with dizziness, speech disturbances, and glassy eyes. Upon hospital admission, swelling and erythema around the cuts were noted, which spread over his whole arm over the next 20 hours. An electrocardiogram detected incomplete left bundle block. Bloodwork was normal, with the exception of slightly elevated levels of creatine kinase, lactate dehydrogenase, and C-reactive protein. He was treated with infusion of physiological fluids. Electrocardiogram changes receded in 24 hours, but paresthesias, weakness, and myalgia persisted for 48 hours until discharge.

Several cases of pulmonary exposure to palytoxin have occurred during the aquaria cleaning using boiling water[78] or otherwise handling *Palythoa*.[79] Bernaconi et al[79] reported on three individuals handling *Palythoa* in a new aquarium to which sea salt had been added, which produced some foam and mist and resulted in pulmonary effects consisting of a restrictive ventilatory pattern with significant hypoxia. Bernasconi et al[79] also reported on an entire family that became ill after a professional aquarium cleaner poured boiling water on a *Palytoxin*-encrusted coral fragment. The cleaner, along with four members of the family elsewhere in the room, reported to the emergency department complaining of respiratory symptoms. All patients developed a low-grade fever, increased white cell

count, and elevated creatine phosphokinase during their hospital stay. The intoxication of other people not directly involved with the aquarium cleaning process strongly suggests extremely high potency of the aerosolized toxic material.

Diagnosis

Context is key to the clinical diagnosis of the known palytoxin intoxication syndromes. Palytoxin poisoning should be suspected in patients who present with rapid onset of respiratory distress and tonic muscle contractions soon after eating grazing fish species (especially sardines, but also other fish such as herring, anchovies, etc), and especially during warm summer months.[80] Essentially the same high fatality syndrome is described in cases of xanthid crab ingestion. Cases have been described only in Caribbean, African coastal, and Indo-Pacific waters. The complication of rhabdomyolysis, with creatine kinase levels peaking at about 24 to 36 hours after symptom onset, would also be an important later clue if the diagnosis has not yet been made.[55,81] Paresthesias and numbness, as well as gastrointestinal symptoms, also occur in the other seafood toxidromes, but absence of frank paresis or paralysis—which has not been described with palytoxins—may be a relative discriminator from PSP. Other palytoxin intoxication syndromes differ from fish poisoning in varying degrees according to the route of exposure: dermal exposure, as with marine aquarium hobbyists, can be expected to produce local and systemic skin manifestations as described above; inhalational exposure near an algal bloom is known to produce respiratory distress and a mild dermatitis, but also fever (in a majority of cases) and conjunctivitis (in a minority). Gastrointestinal symptoms were also reported in a significant minority of inhalational cases, and low-grade fever was reported in most of the aquarium hobbyists.

Definitive laboratory identification of palytoxins in seafood can be accomplished with rapid and sensitive hemolysis neutralization assays,[82] but the relative rarity of these syndromes does not make the licensing or widespread commercial availability of these tests likely in the foreseeable future. A number of other investigational methods of detection (including immunoassays and a fluorescence polarization) have been described in the literature.[62]

Medical Management

No specific therapy exists for palytoxin intoxications. For exposures restricted to contact with intact skin/mucosa only (eg, beachgoers exposed to algal blooms), experience indicates that nonsteroidal anti-inflammatory drugs are effective and that the condition is self-limited.[75] For recent ingestions, however, gastric emptying procedures, implemented as early as possible, are highly desirable and may be undertaken even with uncertainty as to the specific seafood-related toxin involved. Supportive care and the amelioration of symptoms are then the basis of treatment, with aggressive hydration via infusion of intravenous fluids being the primacy focus in significant intoxications where avoidance of rhabdomyolysis-induced renal failure is the goal. Palytoxin(s) have been implicated in a number of human fatalities involving ingestion of tropical fishes, with one author estimating a case fatality rate—based on an admittedly limited number of cases—of 45%.[80] Cardiac conduction disorders and heart failure (with consequent hypotension), as well as renal failure, appear to be important mechanisms in these patients.[80,83]

As with other toxic marine aerosols, the respiratory symptoms associated with inhalation would be expected to rapidly diminish upon vacating the contaminated site, but severe intoxications by this route may require standard treatment for reactive airway disease (nebulized albuterol, anticholinergics, antihistamines).[78] Aggressive treatment in an intensive care facility may prove necessary, and—although not validated—administration of steroids,[79] antihistamines, or benzodiazepines[76] have proved helpful and may be tried.

Given the apparent potency of exposures to aerosols, as well as the lethality associated with ingestions, palytoxins seem to have potential as bioterrorism agents. The large numbers sickened (more than 200 individuals) in the 2005 Genoa event (20% of whom were hospitalized) indicate these possibilities.

SUMMARY

Exposure to marine algal toxins may occur via ingestion or delivery as an aerosol at the tactical level. Although the toxins may be highly lethal, extracting and weaponizing them is relatively difficult because of the relatively small amounts of toxins typically produced by the source organisms. Such toxins may be more suitable for causing incapacitation or death among small groups or for assassinations. The toxins presented in this chapter are diverse in structure and mode of action. Proper diagnosis and care represent a daunting challenge for physicians.

STX, brevetoxins, and domoic acid are marine algal toxins associated with human illness in natural outbreaks related to harmful algal blooms. STX blocks ionic conductance of the voltage-dependent sodium channels, leading to neurological symptoms (parasthesias and paralysis) as well as respiratory distress and cardiovascular instability. Treatment includes respiratory support and intensive cardiovascular management. Anti-STX serum and antibodies have shown promise in animal models, but such reagents are unavailable for human use. Brevetoxins inhibit sodium channel inactivation, leading to depolarization of membranes. Brevetoxin symptoms are similar to those of STX but are usually milder and lack paralysis. Although naturally acquired cases typically resolve spontaneously in 1 to 3 days, patients should be carefully observed and may require aggressive airway management. Domoic acid is a neuroexcitatory amino acid that kills cells within the central nervous system, particularly in the hippocampus, which is associated with learning and memory. Patients with domoic acid intoxication develop gastrointestinal symptoms and neurological symptoms, including anterograde memory loss and myoclonus. Severe intoxications may lead to convulsions and death. Medical management of domoic acid intoxications includes monitoring of hemodynamic status and pharmacological treatment of seizures.

Palytoxins derive from both dinoflagellate blooms or from the polyps of soft corals of the genus *Palythoa*. Their primary mechanism of action is to bind to the Na^+/K^+-ATPase, converting it to a nonselective cationic pore and interfering with intracellular ionic homeostasis. Oral intoxication can lead to malaise, vomiting and diarrhea, a bitter or metallic taste in the mouth, muscle aches and cramps, numbness or tingling in the extremities, bradycardia, and dyspnea. Renal failure resulting from myoglobinemia secondary to rhabdomyolysis can occur. Inhalation exposure can lead to respiratory symptoms such as fever, sore throat, cough, dyspnea, headache, nausea/vomiting, rhinorrhea, lacrimation, and dermatitis. An underappreciated exposure mechanism is through the home aquarium trade, whereby several incidences of inhalational or dermal intoxication have occurred through the handling or disinfection of *Palythoa* polyps. Treatment of palytoxin intoxication, which is nonspecific, is based on the route of exposure. The apparent extreme aerosol toxicity of palytoxins, as suggested by several incidences of beachgoers being intoxicated during near-shore blooms of *O ovata*, makes it critical for further investigation of the aerosol toxicology of these compounds.

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Chapter 20

ALPHAVIRUS ENCEPHALITIDES

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INTRODUCTION

During the 1930s, three distinct but antigenically related viruses recovered from moribund horses were shown to be previously unrecognized agents of severe equine encephalitis. Western equine encephalitis virus (WEEV) was isolated in the San Joaquin Valley in California in 1930[1]; Eastern equine encephalitis virus (EEEV) was isolated in Virginia and New Jersey in 1933[2,3]; and Venezuelan equine encephalitis virus (VEEV) was isolated in the Guajira peninsula of Venezuela in 1938.[4] By 1938, it was clear that EEEV and WEEV were also natural causes of encephalitis in humans,[5–7] and naturally acquired human infections with VEEV occurred in Colombia in 1952 in association with an equine epizootic.[8]

Although these viruses cause similar clinical syndromes in horses, the consequences of the infections they cause in humans differ. Eastern equine encephalitis (EEE) is the most severe of the arboviral encephalitides, with case fatality rates of 30% to 70%, and neurological sequelae common in survivors.[9] WEEV appears to be less neuroinvasive but has pathology similar to that of EEE in patients with encephalitis. In contrast, severe encephalitis resulting from VEEV is rare in humans except for children. In adults, VEEV usually causes an acute, febrile, and incapacitating disease with prolonged convalescence.

The three viruses are members of the *Alphavirus* genus of the family *Togaviridae*. As with most of the alphaviruses, VEEV, EEEV, and WEEV are transmitted by mosquitoes, and are maintained in enzootic cycles with various vertebrate hosts. Thus, the natural epidemiology of these viruses is controlled by environmental factors that affect the interactions of the relevant mosquito and reservoir host populations. Of the 31 viruses currently classified within this group, VEEV, EEEV, and WEEV are the only viruses regularly associated with encephalitis. Although these encephalitic viruses are restricted to the Americas, as a group, alphaviruses have worldwide distribution and include other epidemic human pathogens. Among those pathogens, chikungunya virus (Asia, Africa, and the Americas), Mayaro virus (South America), o'nyong-nyong virus (Africa), Ross River virus (Australia and Oceania), and Sindbis virus (SINV; Africa, Europe, and Asia) can cause an acute febrile syndrome often associated with debilitating polyarthritic symptoms.

Although natural infections with the encephalitic alphaviruses are acquired by mosquito bite, these viruses are also highly infectious by aerosol. VEEV has caused more laboratory-acquired disease than any other arbovirus. Since its initial isolation, at least 150 symptomatic laboratory infections have been reported, most of which were known or thought to be aerosol infections.[10] Before vaccines were developed, most laboratories working with VEEV reported disease among their personnel. The ability of aerosolized EEEV and WEEV to infect humans is less certain, relying on anecdotal evidence and animal studies. EEEV and WEEV are less commonly studied in the laboratory than VEEV, which may explain the lower incidence of laboratory-acquired infections. Therefore, fewer human exposures have occurred or the infectious dose is higher resulting in fewer incidences.

Perhaps as a consequence of their adaptation to dissimilar hosts in nature, the alphaviruses replicate readily and generally to very high titers in a wide range of cell types and culture conditions. Virus titers of 1 billion infectious units per milliliter of culture medium are not unusual, and the viruses are stable in storage and in various laboratory procedures. Because they can be easily manipulated in the laboratory, these viruses have long served as model systems by which to study various aspects of virus replication, pathogenesis, induction of immune responses, and virus–vector relationships. As a result, the alphaviruses are well described and their characteristics well defined.[9,11,12]

The designers of offensive biological warfare programs initiated before or during World War II[13] recognized that the collective in vitro and in vivo characteristics of alphaviruses, especially the equine encephalitis viruses, lend themselves well to weaponization. Although other encephalitic viruses could be considered as potential weapons (eg, the tickborne encephalitis viruses), few possess as many of the required characteristics for strategic or tactical weapon development as the alphaviruses:

- These viruses can be produced in large amounts in inexpensive and unsophisticated systems.
- They are relatively stable and highly infectious for humans as aerosols.
- Strains are available that produce either incapacitating or lethal infections.
- The existence of multiple serotypes of VEEV, as well as the inherent difficulties of inducing efficient mucosal immunity, confound defensive vaccine development.

The equine encephalitis viruses remain as highly credible threats, and intentional release as a small-particle aerosol from a single airplane could be expected to infect a high percentage of individuals within an area

of at least 10,000 km^2. Furthermore, these viruses are readily amenable to genetic manipulation by modern recombinant DNA technology. This characteristic is being used to develop safer and more effective vaccines,[14,15] but, in theory, it could also be used to increase the weaponization potential of these viruses.

HISTORY AND SIGNIFICANCE

Descriptions of encephalitis epizootics in horses thought to have been caused by EEEV were recorded as early as 1831 in Massachusetts.[16] However, it was not until the outbreaks of EEE in Delaware, Maryland, and Virginia in 1933 and 1934 that the virus was isolated, and not until a similar outbreak in North Carolina in 1935 that birds were suspected as the natural reservoir.[17] The initial isolation of EEEV from a bird[18] and from *Culiseta melanura* mosquitoes,[18] the two major hosts of the EEEV natural cycle, were both reported in 1951. Outbreaks of EEEV have occurred in most eastern states and in southeastern Canada, but they have been concentrated along the eastern and Gulf coasts. Although only 270 cases of EEE in humans were reported between 1964 and 2010 (http://www.cdc.gov/easternequineencephalitis/tech/epi.html), the social and economic impact of this disease has been larger than expected because of the high case fatality rate, significant long-term sequelae among survivors, equine losses, extreme concern among individuals living in endemic areas during outbreaks, and the surveillance and mosquito-control measures required. Isolation of EEEV from *Aedes albopictus* mosquitoes, which are prevalent in EEE endemic areas in the United States, has heightened concern because the opportunistic feeding behavior of these mosquitoes and their apparent high vector competence for EEEV suggest that they may be efficient bridge vectors for spillover infections of humans.[19]

The initial isolation in 1930 of WEEV from the brain tissues of a horse with encephalitis was made in the midst of a large epizootic in California, which involved at least 6,000 horses and with an approximate mortality of 50%.[1] Cases of human encephalitis in California were not linked to WEEV until 1938, when the virus was isolated from the brain of a child. During the 1930s and 1940s, several other extensive epizootics occurred in western and north-central states, as well as in Saskatchewan and Manitoba in Canada, which affected large numbers of equids and humans. For example, it has been estimated that during 1937 and 1938, more than 300,000 equids were infected in the United States, and in Saskatchewan, 52,500 horse infections resulted in 15,000 deaths.[20,21] Unusually high numbers of human cases were reported in 1941: 1,094 in Canada and 2,242 in the United States. The attack rate in these epidemics ranged from 22.9 to 171.5 per 100,000, with case fatality rates of 8% to 15%.[21]

In the early 1940s, workers isolated WEEV from *Culex tarsalis* mosquitoes[22] and demonstrated the presence of specific antibodies to WEEV in birds,[23] suggesting that birds are the reservoirs of the virus in nature. The annual incidence of disease in both equids and humans continues to vary widely, which is expected of an arthropodborne disease, and significant epidemics occurred in 1952, 1958, 1965, and 1975.[21]

VEEV was initially isolated during investigations of an epizootic occurring in horses in Venezuela in 1936, and the isolate was shown to be antigenically different from the EEEV and WEEV isolated previously in the United States.[4,24] Over the following 30 years, many VEEV outbreaks were reported among horses, and humans became infected in large numbers in association with these epizootics.[25] Most of those infected recovered after suffering an acute, febrile episode, but abortions and stillbirths were observed in pregnant women and severe disease with encephalitis and death also occurred, mostly in children and older individuals. In the 1960s, major epizootics occurred in Venezuela, Colombia, Peru, and Ecuador, and spread to Central America in 1969.[26] These epizootics and previous ones were associated with significant human suffering, especially among rural people, who suffered not only from disease, but also from the loss of their equids, which were essential for transportation and agriculture. Between 1969 and 1971, epizootics were reported in essentially all of Central America and subsequently continued north to Mexico and into Texas. The most recent major epizootic occurred in Venezuela and Colombia in 1995.[27]

Between active epizootics it was not possible to isolate the equine virulent viruses. During the 1950s and 1960s, however, several other attenuated, antigenically different VEEV strains were isolated from different geographical areas. These enzootic strains could be differentiated antigenically not only among themselves but also from the epizootic strains.[28] Enzootic strains used different mosquito vectors than the epizootic strains,[29] and most used rodents as reservoir hosts.[30] However, despite apparent avirulence for equids, at least some of the enzootic strains caused human disease.[31]

Laboratory studies with EEEV, WEEV, and VEEV quickly and often inadvertently demonstrated how easily these viruses could cause disease when inhaled. In 1943, eight cases of VEE in laboratory personnel resulted from aerosolization of the virus from contaminated

animal caging.[32] In 1959, two reports from the former Soviet Union detailed an incident in which nine vials of VEEV were dropped in a stairwell infecting 24 people, including people who worked on adjacent floors.[33,34] Before the vaccine and more sophisticated personal protective measures were developed, VEEV was among the most common laboratory-acquired infections.[35] It was primarily because of laboratory-acquired infections that it was realized that the current investigational new drug (IND) vaccines may not protect well against aerosol exposure to enzootic strains of VEEV.[36,37] Two fatal laboratory accidents involving WEEV were reported in the late 1930s, one involving a centrifuge accident and another in which the route of infection was unknown.[38,39] Other reports of laboratory-acquired infection of WEEV were not fatal.[40,41] Although EEEV is considered the most virulent of the encephalitic alphaviruses, before 1967 only two cases of laboratory-acquired EEE occurred, neither of which was fatal.[35] Experimental studies in mice, hamsters, rats, and nonhuman primates (NHPs) have all corroborated the disease potential of VEEV, WEEV, and EEEV when aerosolized and inhaled.[42]

Therefore, within 30 years of the initial isolation of EEEV, WEEV, and VEEV, an essentially accurate picture had emerged with respect to their endemic and epidemic behavior, arthropod vectors, reservoir hosts, potential for infection via the respiratory tract, and the diseases produced. Although not then understood at the molecular level, these three viruses were well described as agents of disease, and the basic methods for their manipulation and production were known. The development of this knowledge occurred during the same period of war and political instability that fostered the establishment of biological warfare programs in the United States[43] and elsewhere, and it was evident that the equine encephalitis viruses were optimal candidates for weaponization. The viruses were incorporated into these programs for both potential offensive and defensive reasons. The offensive biological warfare program in the United States was disestablished in 1969 and all stockpiles were destroyed[13] by executive order, which stated:

> The United States shall renounce the use of lethal biological agents and weapons and all other methods of biological warfare. The United States shall confine its biological research to defensive measures such as immunization and safety measures.[44]

Continuing efforts within the US defensive program in the 1960s and 1970s produced four vaccines for the encephalitis viruses: live-attenuated (TC-83) and formalin-inactivated (C84) vaccines for VEEV, and formalin-inactivated vaccines for EEEV and WEEV. These vaccines are used under US Food and Drug Administration IND status for at-risk individuals, distributed under the provisions of the IND, and recommended for use by any laboratory working with these viruses.[10] Although these vaccines are useful, they have certain disadvantages (which are discussed later in this chapter), and next-generation vaccines are being developed.[14]

ANTIGENICITY AND EPIDEMIOLOGY

Antigenic and Genetic Relationships

The three American equine encephalitides antigenic complexes, VEE, EEE, and WEE, have been grouped with eight additional virus complexes into the *Alphavirus* genus based on their serologic cross-reactivity (Table 20-1).[9,45] Analysis of structural gene sequences obtained from members of the VEEV and EEEV complexes confirms the antigenic classification for the most part and serves as another tool for classifying these viruses. Viruses of the WEE complex, including Highlands J, Fort Morgan, and WEEV, have been identified as recombinant viruses originating from ancestral precursors of EEEV and Sindbis virus and fall into a unique genetic grouping of alphaviruses.[46–49]

Venezuelan Equine Encephalitis Complex

The VEE complex consists of eight closely related viruses that manifest different characteristics with respect to ecology, epidemiology, and virulence for humans and equids (Table 20-2). The IA/B and IC varieties are commonly referred to as epizootic strains. These strains, which have been responsible for extensive epidemics in North, Central, and South America, are highly pathogenic for humans and equids. All epizootic strains are exotic to the United States and have been isolated only twice since 1973.[50–52] Enzootic strains include Everglades (formerly subtype II), Mucambo (formerly subtype IIIA), Pixuna (formerly subtype IV), Cabassou (formerly subtype V), Rio Negro (formerly subtype VI), and varieties ID, IE, and Mosso das Pedras (formerly subtype IF).[53–59] Like the epizootic strains, the enzootic strains may cause disease in humans, but they differ from the epizootic strains in their lack of virulence for equines. Infection of equids with some enzootic subtypes leads to an immune response capable of protecting the animals from challenge with epizootic strains.[60] Limited data, acquired following laboratory exposures, suggest that cross-protection between epizootic and enzootic strains may be much less pronounced in humans.[37,61,62]

TABLE 20-1

ANTIGENIC CLASSIFICATION OF ALPHAVIRUSES

	Virus		
Antigenic Complex	**Species**	**Subtype**	**Variety**
Western Equine Encephalitis (WEE)	WEE virus		
	Highlands J virus		
	Fort Morgan virus	Buggy Creek	
	Aura virus		
	Whataroa virus		
	Sindbis virus	Ockelbo	
		Babanki	
		Kyzylagach	
Venezuelan Equine Encephalitis (VEE)	VEE virus	I	A-B
		I	C
		I	D
		I	E
	Mosso das Pedras virus		
	Everglades virus		
	Mucambo virus	Mucambo (IIIA)	
	Tonate virus	Tonate (IIIB)	
		Bijou Bridge (IIIB)	
		71D-1252 (IIIC)	
	Pixuna virus		
	Cabassou virus		
	Rio Negro virus		
Eastern Equine Encephalitis (EEE)	EEE virus		
	Madariaga virus	Madariaga II	
		Madariaga III	
		Madariaga IV	
Semliki Forest	Semliki Forest virus		
	Bebaru virus		
	Chikungunya virus	Chikungunya	Several
	O'nyong-nyong virus	Igbo Ora	
	Getah virus	Getah	
	Ross River virus	Sagiyama	
	Mayaro virus	Mayaro	
	Una virus		
Middelburg	Middelburg virus		
Nduma	Nduma virus		
Barmah Forest	Barmah Forest virus		
Trocara	Trocara virus		
Southern elephant seal	Southern elephant seal virus		
Eilat	Eilat virus		
Salmon pancreas disease	Salmon pancreas disease virus		1-6
			Sleeping disease

Data sources: (1) Nasar F, Palacios G, Gorchakov RV, et al. Eilat virus, a unique alphavirus with host range restricted to insects by RNA replication. *Proc Natl Acad Sci U S A*. 2012;109:14622–14627. (2) King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, eds. *Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses*. San Diego, CA: Elsevier; 2012.

Eastern Equine Encephalitis Virus

The EEEV complex previously consisted of viruses in two antigenically distinct forms: (1) the North American and Caribbean (NA EEEV), and (2) the South American (SA EEEV). A recent proposal accepted by the International Committee on Taxonomy of Viruses resulted in the reclas-

TABLE 20-2

THE VENEZUELAN EQUINE ENCEPHALOMYELITIS COMPLEX

Subtype	Variety	Prototype Strain	Origin	Cycle	Disease in Horse	Disease in Man
VEEV	IA/B	Trinidad donkey	Donkey (Trinidad)[1]	Epizootic	+	+
	IC	P-676	Horse (Venezuela)[2]	Epizootic	+	+
	ID	3880	Human (Panama)[3]	Enzootic	–	+
	IE	Mena II	Human (Panama)[1]	Enzootic	–	+
Mosso das Pedras virus		78V-3531	Mosquito (Brazil)[4]	Enzootic	–	?
Everglades virus		Fe3-7c	Mosquito (Florida)[5]	Enzootic	–	+
Mucambo virus		Mucambo (BeAn8)	Monkey (Brazil)[6]	Enzootic	–	+
Tonate virus	IIIB	Tonate (CaAn410-D)	Bird (French Guiana)[7]	Enzootic	–	+
	IIIC	71D-1252	Mosquito (Peru)[8]	Enzootic	–	?
Pixuna virus		Pixuna (BeAn356445)	Mosquito (Brazil)[6]	Enzootic	–	?
Cabassou virus		Cabassou	Mosquito (French Guiana)[7]	Enzootic	–	?
Rio Negro virus		AG80-663	Mosquito (Argentina)[9]	Enzootic	–	+

Sources that contain original descriptions of or additional information about this strain: (1) Young NA, Johnson KM. Antigenic variants of Venezuelan equine encephalitis virus: Their geographic distribution and epidemiologic significance. *Am J Epidemiol*.1969;89:286. (2) Walton TE. Virulence properties of Venezuelan equine encephalitis virus serotypes in horses. In: Venezuelan Encephalitis: Proceedings of the Workshop-Symposium on Venezuelan Encephalitis Virus, Washington, DC, 14–17 Sep 1971. Washington, DC: Pan American Health Organization; 1972:134. PAHO Scientific Publication 243. (3) Johnson KM, Shelokov A, Peralta PH, Dammin GJ, Young NA. Recovery of Venezuelan equine encephalomyelitis virus in Panama: a fatal case in man. *Am J Trop Med Hyg*. 1968;17:432–440. (4) Walton TE, Grayson MA. Venezuelan equine encephalitis. In: Monath TP, ed. *The Arboviruses: Epidemiology and Ecology*. Vol 4. Boca Raton, FL: CRC Press; 1988:203–231. (5) Chamberlain RW, Sudia WD, Coleman PH, Work TH. Venezuelan equine encephalitis virus from South Florida. *Science*. 1964;145:272. (6) Shope RE, Causey OR, de Andrade AHP, Theiler M. The Venezuelan equine encephalitis complex of group A arthropod-borne viruses, including Mucambo and Pixuna from the Amazon region of Brazil. *Am J Trop Med Hyg*. 1964;13:723. (7) Karabatsos N. *International Catalogue of Arboviruses Including Certain Other Viruses of Vertebrates*. 3rd ed. San Antonio, TX: American Society for Tropical Medicine and Hygiene; 1985. (8) Scherer WF, Anderson K. Antigenic and biological characteristics of Venezuelan encephalitis virus strains including a possible new subtype isolated from the Amazon region of Peru in 1971. *Am J Epidemiol*. 1975;101:356. (9) Contigiani MS, De Basualdo M, Camara A, et al. Presencia de anticuerpos contra el virus de la encefalitis equina Venezolana subtipo VI en pacientes con enfermedad aguda febril [in Spanish]. *Revista Argentina de Microbiologia*. 1993;25:212–220.
Adapted with permission from Walton TE, Grayson MA. Venezuelan equine encephalitis. In: Monath TP, ed. *The Arboviruses: Epidemiology and Ecology*. Vol 4. Boca Raton, FL: CRC Press; 1989: 206.

sification of the South American variants as a distinct species, Madariaga virus (MADV), within the *Alphavirus* genus.[63,64] Thus, EEEV will refer to the former NA EEEV, whereas the new species designation, MADV, will refer to the former SA EEEV. The two species can be distinguished readily by hemagglutination-inhibition (HI) assays and plaque-reduction neutralization tests.[63,65] All EEEV isolates show a high degree of genetic and antigenic homogeneity. However, they are distinct from the MADV isolates, which are more heterogeneous and form three genetic subtypes (II, III, IV).[66,67] EEEV strains are extremely virulent for humans and horses. Although MADV subtypes infect both horses and humans, infections are rarely associated with significant clinical disease.[68,69] However, a MADV subtype III virus (ArgM) resulted in up to 75% lethality, depending on the dose, in rodent models infected by aerosol.[70]

Western Equine Encephalitis Complex

Six virus species, including WEE, Sindbis, Aura, Fort Morgan, Highlands J, and Whataroa, comprise the WEEV complex.[9,64] Several antigenic subtypes of WEEV have been identified, but their geographical distributions overlap.[52] Most of the members of the WEE complex are distributed throughout the Americas, although Whataroa and subtypes of SINV have strictly Old World distributions.[9,12] The New World WEE complex viruses can be distinguished readily by neutralization tests. In addition, WEE complex viruses isolated in the western United States (eg, WEEV) are genetically distinct from those commonly found in the eastern United States (eg, Highlands J).[67,71] SINV is considered a member of the WEEV complex based on antigenic relationships. However, sequence comparisons show that WEEV, Highlands J, and Fort Morgan are actually derived from a

recombination event between ancestral SINV and EEEV (or MADV). The structural domains of the recombinant viruses were derived from the SINV ancestor, whereas the nonstructural domains were derived from the EEEV ancestor.[67,72]

Epidemiology and Ecology

The evolution of the equine encephalitides is closely tied to the ecology of these viruses in naturally occurring endemic foci. Evidence indicates that the relative genetic homogeneity of the EEE and WEE complex viruses may result from the mixing of virus subpopulations as a result of the movement of the virus from one location to another by the avian hosts.[73] In general, these viruses are maintained in a consistently virulent state, capable of initiating epizootics without development of any significant mutations. In contrast, diversity within the VEEV complex results from local evolution of these viruses in mammalian hosts that live in defined habitats. Initiation of epizootic and epidemic activity is almost always associated with appearance of specific genetic change.[73]

Most commonly, human involvement in the form of endemic and epidemic activity, occurs following intrusion into geographical regions where natural transmission cycles are occurring or following perturbation of these cycles by environmental changes or the addition of other vectors.[74] The dramatic exception to this is epizootic VEEV, in which the spreading waves of the epizootic among equines can move rapidly over large distances, and humans become infected by mosquitoes that have fed on viremic equines. The high levels of viremia in equines infected with epizootic VEEV make them efficient amplifying hosts, with the result that equine infections normally precede human infections by days to weeks.[75] Recent evidence suggests that it is the adaptation of these enzootic subtype ID viruses for efficient replication in horses that leads to emergence and efficient epidemic spread of disease.[19,76] Medical personnel should view with some suspicion evidence of widespread human VEEV infections outside of endemic areas, in the absence of mosquito vectors or in the absence of equine disease, because this combination of circumstances may indicate an unnatural release of virus into the environment.

Enzootic VEEV subtypes, as described above, are maintained efficiently in transmission cycles involving primarily rodents and *Culex* mosquitoes belonging to the subgenus *Melanoconion*.[77–79] These mosquitoes live in humid locales with abundant open spaces such as sunny, swampy pastures cut by slowly flowing streams. The mosquitoes are ground feeders, seldom found higher than 8 meters above ground, and some prefer feeding on mammals rather than birds.[80] Ground-dwelling rodents, partly because their ecologies are similar to that of the mosquito vectors, are the primary vertebrate hosts for the enzootic forms of VEEV. Following infection, these animals develop viremia of sufficient magnitude and duration to infect mosquitoes feeding on their blood.[81] Other animals, such as bats and certain birds, may play a secondary role.[82] Seroprevalence rates among human populations living in or near endemic VEEV areas vary but can approach 100%, suggesting that continuous transmission occurs presumably in the absence of significant human disease.[75] However, virus activity within endemic zones can also be highly focal. In one incident at the Fort Sherman Jungle Operations Training Center in the Panama Canal Zone in December 1967, 7 of 12 US soldiers camped in one area developed VEE disease within 2 days, but another group that camped only a few yards away showed no disease.[83,84] The incidence of human disease during epizootics also varies, but it is often high. During an outbreak in Venezuela, attack rates of 119 per 1,000 inhabitants per month were reported.[85] Following an epizootic in Guatemala and El Salvador, overall seroprevalence was estimated at 20%.[86]

Unlike the enzootic strains, the fate of the epizootic strains during interepidemic periods is unclear. The most appealing theory on how epizootic strains arise suggests that they evolve by mutation and equine selection from enzootic strains. Results from oligonucleotide fingerprinting and sequence analysis of ID isolates from Colombia and Venezuela reveal a close similarity to the epizootic strains, suggesting that the equine virulent epizootic strains arise naturally from variants present in populations of ID virus.[87,88]

Although the genetic evidence indicates that mutation of enzootic strains may lead to the development of epizootic strains, ecological data suggest a strong selective pressure to maintain the enzootic genotype in certain habitats. The enzootic VEEV vector *Culex* (*Melanoconion*) *taeniopus* is fully susceptible to both IAB and IE strains following intrathoracic inoculation. Orally exposed mosquitoes are fully competent vectors of the enzootic strain; however, they fail to develop disseminated infection or transmit epizootic virus.[29,89] In the absence of genetic change, this virus–host interaction appears to be relatively stable. Mosquito resistance to epizootic strains of VEEV is rare. Epizootic strains have been isolated from a large number of mosquito species, and many have been shown to be efficient vectors.[90] Thus, host switching from enzootic to epizootic vectors may be an important factor in the evolution of epizootic VEEV strains. Researchers suggest that emer-

gence of epizootic strains may result from acquisition of mutations that allow for transmission by abundant, equiphilic mosquitoes. More specifically, adaptation to *Aedes* (*Ochlerotatus*) *taeniorhynchus* mosquitoes has been a determinant of some recent emergence events, providing further evidence that the ability to switch hosts is critical for emergence of epizootic strains.[76] The introduction of mosquito species into previously unoccupied geographical ranges (eg, *Aedes albopictus* into North America) may, therefore, offer the opportunity for epizootic strains to reemerge.

A major outbreak of epizootic VEEV occurred in the late 1960s and early 1970s. Epizootic virus first reached North America in 1969,[25] but did not reach the United States until 1971. Studies of this epizootic demonstrated that the virus easily invaded territories in which it was formerly unknown,[85] presumably as a result of (*a*) the availability of large numbers of susceptible equine amplifying hosts and (*b*) the presence of competent mosquito vectors. The initial outbreak in North America, attributed to enzootic strain IE, occurred in 1966 in Tampico, Mexico, involving approximately 1,000 equids.[91]

By the end of 1969 and the beginning of 1970, the expansion of the outbreak prompted the Mexican government to request the TC-83 vaccine from the US Army through the US Department of Agriculture.[92] Despite the immunization of nearly 1 million equids, the epizootic continued to spread and reached the United States in June 1971. The nature of the virus and the number of human and equine cases prompted the US Secretary of Agriculture to declare a national emergency on July 16, 1971.[93] Subsequent immunization of more than 2 million horses and unprecedented mosquito abatement efforts eventually stopped the epizootic before it was able to spread from Texas. Epizootic VEEV has not been isolated in the United States since the 1971 outbreak.

The first large outbreak since the 1969–1971 epizootic occurred in 1995 (Figures 20-1 and 20-2). The epizootic began in northwestern Venezuela and spread across the Guajira peninsula into northeastern Colombia. An estimated 75,000 to 100,000 humans were infected, with more than 20 deaths reported. This outbreak was caused by a VEEV IC strain. By sequence analysis, this strain proved to be essentially identical to a virus that caused an outbreak in Venezuela in 1962–1964.[27] Outbreaks of traditionally enzootic strains of VEEV also have



Figure 20-1. This photograph was taken in 1995 near Buena Vista, Colombia. During large Venezuelan equine encephalitis (VEE) epizootics, typical morbidity rates among unvaccinated equines are 40% to 60%, with at least half of the affected animals progressing to lethal encephalitis. Note the disruption of the ground surface, which is caused by the characteristic flailing or swimming syndromes of moribund animals. Although clinically indistinguishable from the syndromes produced by eastern equine encephalitis and western equine encephalitis viruses, the capability of VEE to initiate explosive and rapidly expanding epizootics makes reliable diagnostic tests essential for the initiation of appropriate veterinary and public health measures.



Figure 20-2. This photograph was taken in 1995 near Maicao, Colombia. Equine vaccination is the most effective means available to prevent Venezuelan equine encephalitis (VEE) epizootics as well as to control emerging outbreaks. Equines are the major amplifying hosts, and maintaining a high rate of immunity in the equine population will largely prevent human infection with the epizootic strains of VEE. Both inactivated and live attenuated vaccines are available for veterinary use, but the ability of the live attenuated vaccine to induce immunity in 7 to 10 days with a single inoculation makes it the only practical vaccination strategy in the face of an outbreak. Other measures used to control outbreaks include using insecticides to reduce mosquito populations and prohibiting the transportation of equines from affected areas.

occurred in Mexico and Central America. Genetic analysis confirmed acquisition of mutations and provided further evidence that emergence of epizootic strains may result from the accumulation of genotypic changes in enzootic strains.[94,95]

EEEV is endemic to focal habitats ranging from southern Canada to Central America. The virus, which has been isolated as far west as Michigan, is most common along the eastern coast of the United States between New England and Florida. Enzootic EEEV transmission occurs almost exclusively between passerine birds (eg, the perching songbirds) and the mosquito *Culiseta melanura*. Because of the strict ornithophilic feeding behavior of this mosquito, human and equine disease requires the involvement of more general feeders, such as members of the genera *Culex* and *Coquillettidia*. Recent evidence suggests EEEV may overwinter in the southeastern United States in reptiles or amphibians, further necessitating the participation of more general feeding vectors.[96,97] Mosquito vectors belonging to *Culex* species, subgenus *Melanconion*, may play a role in maintaining and transmitting MADV subtypes.[98]

WEEV is the best studied member of the WEE complex in terms of its epidemiology. The virus is maintained in cycles involving passerine birds and the mosquito *Culex tarsalis*. Humans and equids become infected only tangentially and are considered to be dead-end hosts,[99] indicating that they do not normally contribute to further spread of the virus in nature.

STRUCTURE AND REPLICATION OF ALPHAVIRUSES

Virion Structure

The alphavirus virion, a spherical particle approximately 65 nm to 70 nm in diameter, is typically composed of three structural proteins enclosing a single molecule of single-stranded RNA. The RNA genome is packaged within an icosahedral nucleocapsid, which is constructed from multiple copies of the capsid (C or CP) protein (Figure 20-3). The nucleocapsid is, in turn, surrounded by a lipid envelope derived from areas of the host cell plasma membrane that had previously been modified by the insertion of two viral glycoproteins. These envelope glycoproteins, E1 and E2, form heterodimers that associate further into trimers[100,101] to form the short spikes on the surface of the virion. Although a third glycoprotein, E3, was thought to be absent in the mature virion of most alphaviruses, three-dimensional reconstruction of VEEV virions (TC-83 strain) from cryoelectron microscopic images revealed that E3 is associated with the E1–E2 dimers on the virion surface, but at a lower stoichiometry.[102] Evidence of E3 on the surface of Semliki Forest virus virions has also been reported.[103] However, E1 and E2 dimers—but not E3—are known to be targets of the neutralizing antibody response and are among the determinants of tropism and virulence.[104,105] Although nonneutralizing, a monoclonal antibody directed against the VEEV E3 protein provided complete protection against an intraperitoneal challenge when administered before exposure.[106] It is possible that this monoclonal antibody protects by binding to the E3 protein at the surface of infected cells blocking the ability of the virus to bud.

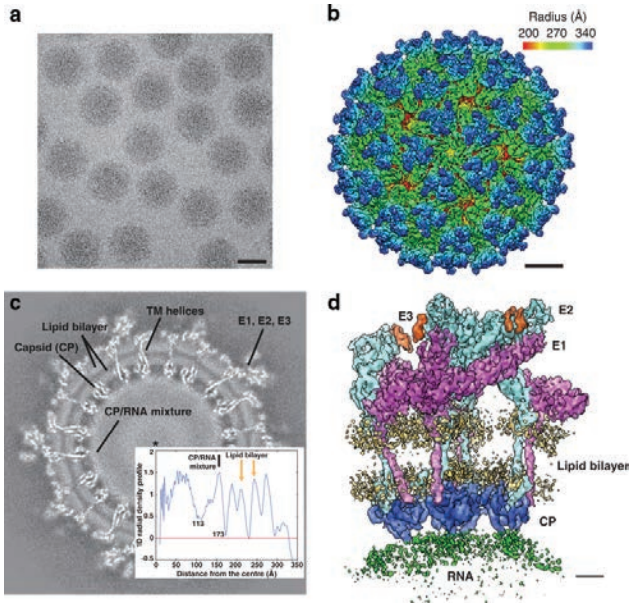


Figure 20-3. 3D structure of Venezuelan equine encephalitis virus (VEEV). (**a**) A typical CCD image of VEEV TC-83 embedded in vitreous ice. Scale bar: 50nm. (**b**) Radially colored 3D reconstruction of VEEV, showing the E1 basal triangle (green) and the E2 central protrusion (blue) for each spike. Scale bar: 10nm. (**c**) A slice through the 3D density map 20 pixels from the origin. The inset is the 1D radial density profile of the map and is aligned to the slice image. (**d**) One asymmetric unit of the virus containing four unique copies of E1 (magenta), E2 (cyan), E3 (orange), and CP (blue). The cryo-EM densities for the viral membrane (yellow) and genomic RNA (green) are also displayed at a slightly lower isosurface threshold. Scale bar: 2nm.
Data source: Zhang R, Hryc CF, Cong Y, et al. 4.4 Å cryo-EM structure of an enveloped alphavirus Venezuelan equine encephalitis virus. *EMBO J*. 2011;30(18):3854–3863. Reproduced with permission from EMBO.

Viral Infection

The infection cycle is initiated when the glycoprotein spikes on the virion bind to receptors on the cell

surface. The virus is localized initially to clathrin-coated pits, where it is engulfed in a coated vesicle and transported to the endosomal compartment within the interior of the cell. A decrease in the pH in the interior of the vesicle induces a conformational change in the glycoprotein spikes, and rearrangement of the E1 glycoprotein mediates fusion of the virion envelope with the endosomal membrane.[107] This fusion results in the release of the nucleocapsid into the cytoplasm, where disassembly of the nucleocapsid releases the viral RNA genome to the synthetic apparatus of the cell.[108]

Genomic RNA

The viral genome, a positive-sense RNA of approximately 11,700 nucleotides, has the structural features of messenger RNA (ie, mRNA, a 5′ methylated cap [m7GpppA] and a poly-A tract at the 3′ end).[109] As a complete and functional mRNA, genomic RNA purified from virions is fully infectious when artificially introduced (ie, transfected) into susceptible cells. Similarly, RNA transcribed from a full-length complementary DNA clone of an alphavirus is also infectious, which allows relatively easy genetic manipulation of these viruses. Mutations introduced into a complementary DNA clone by site-directed mutagenesis are reflected in the RNA transcribed from the altered clone and in the virus produced in transfected cells. These procedures are being used to develop improved vaccines,[14] but they could also be used to enhance specific characteristics required for weaponization.

Structural Protein Synthesis

The alphavirus genome contains two protein coding regions or open reading frames. The 5′ 7,500 nucleotides encode a 220,000-dalton precursor polypeptide, which is proteolytically processed to produce the four components of the viral RNA polymerase. The polymerase genes are followed by a second coding region of approximately 3,800 nucleotides, which contains the information that directs the synthesis of the viral structural proteins. Soon after release of the viral genome from the nucleocapsid, the 5′ 7,500 nucleotides of the genome RNA are translated to produce the viral RNA polymerase. Early in infection, the incoming viral genome is also used as a template for the synthesis of a negative-sense RNA, identical in length to the genome RNA, but of opposite polarity. The negative-sense RNA subsequently serves as a template for the synthesis of additional genomic RNA. The negative-sense RNA is also used as a template for transcription of a capped and polyadenylated subgenomic mRNA, which is identical to the 3′ third of the genome. The subgenomic mRNA is translated to yield a precursor polypeptide that is proteolytically processed by cotranslational and posttranslational cleavages to produce the viral structural proteins. The order of the structural proteins within the precursor is C-E3-E2-6K/TF-E1.

As the subgenomic mRNA is translated, the C protein is produced first and catalyzes its own cleavage from the nascent polypeptide soon after the ribosome transits into the sequences that encode E3. After release of the C protein, the free amino terminus of E3 is bound to the membranes of the rough endoplasmic reticulum. As the synthesis of nascent E3 and E2 (precursor E2 or pE2) continues, the polypeptide is translocated into the lumen of the endoplasmic reticulum, where oligosaccharides and fatty acids are added.[110] A domain of hydrophobic amino acids near the carboxyl terminus of E2 inhibits further transmembranal movement so that the last 30 to 40 amino acids of the E2 polypeptide remain exposed on the cytoplasmic side of the membrane. The 6K polypeptide serves as a signal for membrane insertion of the second glycoprotein, E1, and is subsequently cleaved from both E2 and E1 by signal peptidase.[111] A hydrophobic anchor sequence present near the carboxyl terminus of E1 secures the protein in the membrane.

Budding and Release of Progeny Virus Particles

Soon after synthesis, the precursor of PE2 and E1 interact to form multimeric complexes,[112] which are then transported through the Golgi apparatus, where the final modifications of the oligosaccharide are made. The pE2 protein is cleaved to generate the mature E2 and E3 glycoproteins soon after the glycoproteins leave the Golgi apparatus,[113] and the mature viral spikes assume an orientation in the plasma membrane with the bulk of the E2 and E1 polypeptides exposed on the exterior surface of the cell. In vertebrate cells, final assembly of progeny virus particles happens by budding exclusively at the plasma membrane[114]; whereas in cultured arthropod cells, budding also occurs at intracellular membranes.[115]

In vertebrate cells, budding is initiated when intracellular nucleocapsids bind to the 30 to 40 amino acid cytoplasmic domain of the E2 glycoprotein,[116–118] inducing the formation of a locally ordered array of glycoprotein spikes, which excludes most of the cellular membrane proteins from the region.

Additional lateral associations between the individual spikes stabilize the lattice and promote additional E2–C protein interactions. The growing lattice may draw the membrane around the nucleocapsid, completing the envelopment with the release of the spherical virus particle. Maximal amounts of virus are typically produced from mammalian cells within 8 to 10 hours after infection, and disintegration of the infected cell is likely caused by programmed cell death (apoptosis) rather than direct effects of the virus on cellular function.[119] In contrast, alphaviruses initially replicate to high titer in arthropod cells with little or no evidence of cytopathology. The surviving cells continue to produce lesser amounts of virus, often for weeks or months. The ability of the virus to replicate without causing cell death in arthropod cells may be critical for maintenance of the virus in the mosquito vector in nature.

PATHOGENESIS

In humans, the pathogenesis of VEEV, EEEV, and WEEV infections acquired by aerosol, which is the route of greatest biological defense concern, is unknown. Little is known of the pathogenesis following natural vectorborne infections of humans, mainly because of the limited autopsy material. Much of the information on VEEV pathogenesis in humans is based on a histological review of 21 human fatalities from the 1962–1963 VEEV epidemic in Zulia, Venezuela.[120] With few exceptions, the histopathological lesions in these cases, all among children or young adults, were comparable to those observed in experimentally infected animals. Tissues commonly affected in both humans and animals[121–129] include those of the lymphoid and reticuloendothelial systems as well as the central nervous system (CNS). Widespread hepatocellular degeneration and interstitial pneumonia, not ordinarily seen in experimental animals, were frequent histological findings in these cases of severe human disease. Much of the understanding of the pathogenesis of VEEV, EEEV, and WEEV has relied on animal studies.

The clinical and pathological responses of the host to VEEV, EEEV, and WEEV infection are highly dependent on a number of host and viral factors, including:

- the species, immune status, and age of the host;
- the route of infection; and
- the strain and dose of virus.

Most of the existing experimental data are from studies using rodent models challenged with the virulent Trinidad donkey (TrD) strain of VEEV, an epizootic IAB serotype virus, or its genetic clone V3000. A few NHP studies have also been reported.[130,131] In animal models, as in humans, the lymphatic system and the CNS are consistent target organs. However, the relative degree of injury caused to these tissues varies. Virulent VEEV causes limited and reversible lesions to the lymphoid organs of mice and NHPs,[122,126] but in guinea pigs and hamsters, it causes extreme and irreversible damage to those organs.[127,128] As a result, in the guinea pig and hamster models, death occurs before serious CNS disease develops.[124,125] The host species and the route of administration of VEEV greatly affect CNS disease development. Mice uniformly exhibit a severe paralytic episode before death from diffuse encephalomyelitis following peripheral or aerosol administration of TrD or V3000.[122,126,132,133] NHPs, however, exhibit few if any clinical signs of encephalitis following peripheral inoculation with TrD, and only modest perivascular cuffing and gliosis, mainly in the thalamus, hypothalamus, and olfactory areas of the brain.[122,123] In one study, NHPs inoculated by the intraperitoneal route developed transient viremia and biphasic fever but otherwise exhibited no evidence of clinical disease.[122] NHPs in this study developed brain lesions as early as 6 days postinfection, which typically included lymphocytic perivascular cuffs and gliosis, with the thalamus being the site of the most intense inflammation.[122] NHPs infected by intranasal inoculation had more moderate inflammation, especially in the cortex and hypothalamus,[134] whereas a Colombian epizootic strain of VEEV given by aerosol caused severe clinical and pathological CNS signs and resulted in death in approximately 35% of rhesus macaques.[123] In another study, cynomolgus macaques infected with the VEEV IE or Mucambo virus (IIIA) developed fever, viremia, and mild clinical signs of encephalitis (tremors, loss of coordination) but recovered.[135] Both mice and cynomolgus macaques challenged intracerebrally with TrD or related VEEV strains developed severe and lethal neurological signs with moderate to severe brain histopathology.[134,136]

The mechanisms of neuroinvasion by VEEV represent an important issue, particularly regarding immunoprophylaxis. The specific mechanism of neuroinvasion in the case of peripheral inoculation of virus is not completely understood; however, important features of the process have been elucidated by animal studies. In mice inoculated peripherally and subsequent to the development of viremia, virulent VEEV is detectable in the brain, initially in the olfactory bulbs, and usually within 48 hours of infection.[133,137,138]

It appears that virus in the blood escapes from fenestrated capillaries supplying the olfactory lining of the nasal tract. Virus may then invade olfactory neuron cell bodies or their axons and may be carried via the olfactory nerves into the olfactory bulbs of the brain. However, surgical or chemical ablation of the olfactory lining did not significantly affect the mortality rate or average survival time of infected mice. In this case, neuroinvasion was suspected to occur via the trigeminal nerves.[137] However, direct invasion of the brain across the blood–brain barrier[125,139] seems less compelling than the olfactory route.

The understanding of the mechanism of neuroinvasion following respiratory infection is more clear. In mice, an early and strong target of virulent VEEV administered by aerosol has been shown to be the olfactory neuron.[133] This cell type, a so-called "bipolar neuron," is in direct contact with inspired air at one pole and synapses with resident neurons in the olfactory bulb at the opposite pole, offering a direct connection to the brain independent of viremia development. Both the nasal olfactory epithelium and the olfactory nerve axon bundles in the underlying connective tissue exhibit VEEV antigen within 24 hours of aerosol infection (Figure 20-4), and the olfactory bulbs also show viral infection shortly thereafter (Figure 20-5). In a study of rhesus macaques inoculated intranasally with VEEV, the virus gains access to the olfactory bulb within 24 hours after infection and before the onset of viremia, suggesting direct neuroinvasion via olfactory neurons similar to neuroinvasion in the mouse.[140]

However, in inoculated macaques whose olfactory nerves had been surgically removed, VEEV was still able to reach the olfactory bulb by 36 hours after infection, presumably by the vascular route. Although the olfactory bulb and olfactory tract were sites of early viral replication, the virus did not appear to spread to the rest of the brain along the neural tracts in these monkeys, as it does in mice. In a more recent study in which cynomolgus macaques were exposed to VEEV by the aerosol route, virus was not detected in the brain until 4 days postinfection and was only detected in the region of the olfactory tubercle.[132,141] However, the dose delivered to the cynomolgus macaques in this study was not reported, which could influence the resulting pathology. The teeth are another early target of VEEV administered peripherally or by aerosol,[132,133,137] and the trigeminal nerves appear to carry VEEV from the teeth into the brains as an alternate, although it is probably a less significant route of neuroinvasion.

The pathogenesis of EEEV has not been as thoroughly evaluated. In contrast to VEEV, EEEV replication in lymphoid tissues is limited by tissue-specific microRNA[142]; primary EEEV replication after subcutaneous inoculation occurs in fibroblasts, skeletal muscle, and osteoblasts.[133] In a recent study, it was shown that when mice are infected with EEEV strain FL93-939 by either the aerosol or intranasal route that the virus specifically targets the olfactory epithelium and enters the brain via the olfactory tract.[143,144] In mice exposed by the aerosol route, virus was detected in the brain as early as 6 hours postinfection. In mice inoculated by the subcutaneous route, the mechanism of neuroinvasion

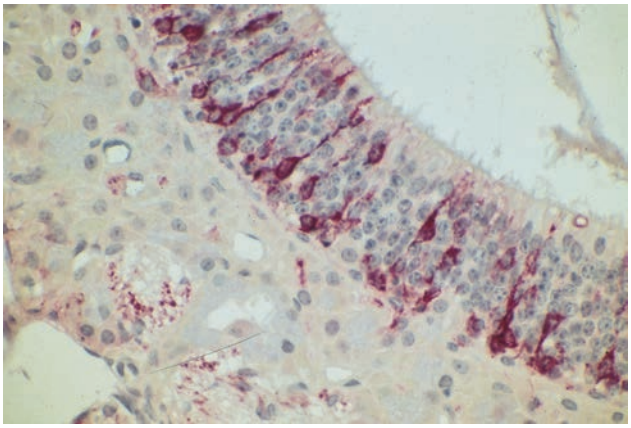


Figure 20-4. Nasal tissue, BALB/c mouse, 2 days after exposure to aerosolized Venezuelan equine encephalitis (VEE) virus. Note immunoreactive olfactory epithelium and olfactory nerves. Alkaline phosphatase-labeled streptavidin method using rabbit antiserum to VEE virus (Mayer's hematoxylin counterstain, original magnification x 300).

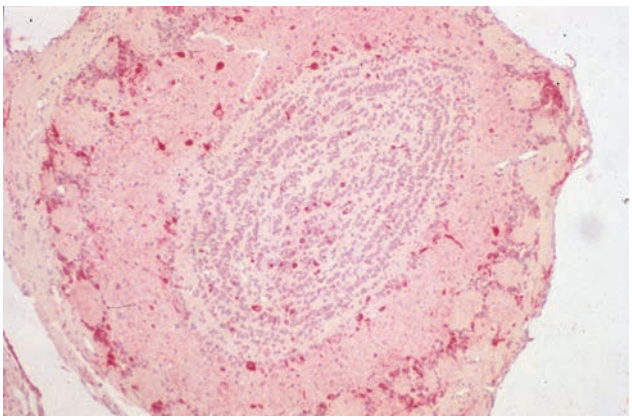


Figure 20-5. Olfactory bulb, BALB/c mouse, 2 days after exposure to aerosolized Venezuelan equine encephalitis (VEE) virus. Note immunoreactive cells. Alkaline phosphatase-labeled streptavidin method using rabbit antiserum to VEE virus (Mayer's hematoxylin counterstain, original magnification x 150).

is less clear and the virus may enter the brain either by the olfactory tract or the vascular route. In all cases, once in the brain, the neuron is the main viral target and animals exhibited varying degrees of neuronal cell death and meningoencephalitis.[143,144]

In a study in which guinea pigs were infected with EEEV by aerosol, animals developed clinical signs within 24 hours of infection and rapidly progressed to include circling, recumbency, coma, and death.[71] No difference in virulence or time to death was seen whether virus was targeted to the lower respiratory tract or upper respiratory tract by manipulating particle size. In these animals, virus was found in the olfactory epithelium and olfactory bulbs followed by spread to all parts of the brain by 4 days postinfection. Again, the neuron was the main viral target and brain lesions included neuronal necrosis, perivascular cuffs, and encephalitis with vasculitis noted in few animals in late-stage cases.[71]

EEEV natural history and pathogenesis studies in NHPs are limited. However, in a recent natural history study cynomolgus macaques were challenged with aerosolized EEEV. In this study, 66% of the animals exhibited fever, leukocytosis, and neurological signs and succumbed 5 to 9 days postchallenge.[145] The major pathological changes in the brain included severe meningoencephalomyelitis characterized by neuronal necrosis, perivascular cuffs, gliosis, satellitosis, edema, hemorrhage, and vasculitis in some animals.[141]

Since there has been a dramatic decline in the incidence of WEEV infection in humans and horses since the middle of the 20th century, few animal studies with this virus have been conducted in recent years. In one study, mice were infected with several strains of WEEV by various routes.[146] The McMillian strain of WEEV was 100% lethal by the intracranial, subcutaneous, and aerosol routes and 90% lethal by the intravenous and intranasal routes. Histopathological lesions occurred in the brains of all mice and were characterized by neuronal necrosis, edema, lymphocytic meningitis, and occasional fibrin thrombi. Two studies in hamsters using various strains of WEEV[147,148] showed lethality by various routes of infection with the major histopathological lesions in the brain being neuronal necrosis, lymphocytic meningitis and perivascular cuffs, astrocytosis, microgliosis, and hemorrhage. In a recent study, cynomolgus macaques were infected with the CBA-87 strain of WEEV by aerosol.[149] Affected animals developed fever, inappetence, lethargy, and tremors, as well as leukocytosis and hyperglycemia. The histopathological lesions were characterized as nonsuppurative meningoencephalomyelitis. In particular, infection was noted in Purkinje cells in the cerebellum and hypothalamus, the region of the brain that controls body temperature.

The specific viral and host mechanisms that contribute to neuroinvasion and neurovirulence for each of these viruses have yet to be elucidated. The importance of these mechanisms and the differences observed between peripheral and aerosol administration are of significant practical concern because the immunological mechanisms of virus neutralization respective to each route can vary greatly, as studies have shown.[130,150,151] The efficiency and rapidity of neuroinvasion following aerosol infection also place very high demands on the vaccines used for immunoprophylaxis (vaccines are discussed later in this chapter).

CLINICAL DISEASE AND DIAGNOSIS

VEEV, EEEV, and WEEV are also recognized for their potential for neuroinvasion and encephalitis in humans, sometimes in epidemic proportions. However, many of the infections caused by these viruses are manifested as systemic viral febrile syndromes, and infections by EEEV and WEEV may remain subclinical. Furthermore, these alphaviruses vary markedly in both their neurotropism and the severity of their neurological sequelae. Depending on the virus, patients presenting with the general syndrome of alphavirus encephalitis have a varying combination of fever, headache, confusion, dysphasia, seizures, paresis, ataxia, myoclonus, and cranial nerve palsies.

Venezuelan Equine Encephalitis

The IAB and IC subtypes of VEEV, which are pathogenic for equines, have the capacity for explosive epizootics with epidemic human disease. Epidemics of VEE affecting 20,000 to 75,000 people have been documented in Colombia, Venezuela, and Ecuador.[25,75,152] In contrast to the other alphavirus encephalitides (EEEV and WEEV), epizootic strains of VEEV are mainly amplified in equids, rather than birds, so that equine disease normally occurs before reports of human disease. Enzootic VEEV strains (subtypes ID and IE, as well as Mosso das Pedras, Everglades, Mucambo, Tonate, Pixuna, Cabassou, and Rio Negro viruses, previously known as VEEV subtypes IF, II, IIIA, IIIB, IV, V, and VI, respectively) are not recognized as virulent for equines, but disease has been documented with most of these variants in humans who reside in or move into enzootic foci, or after laboratory infections (see Table 20-2). The resulting syndromes appear to be similar—if not indistinguishable—from the syndrome produced by epizootic variants, which ranges from undifferentiated febrile illness to fatal encephalitis. In NHPs,

aerosol exposure to enzootic strains results in a febrile illness with indications of encephalitis virtually indistinguishable from that seen with epizootic strains in terms of onset, severity, and duration.[153]

Following an incubation period that can be as short as 28 hours but is usually 2 to 6 days, patients typically develop a prostrating syndrome of chills, high fever (38°C–40.5°C), headache, and malaise.[154] Photophobia, sore throat, myalgias, and vomiting are also common symptoms. Frequent signs noted on physical examination include conjunctival injection, erythematous pharynx, and muscle tenderness. Although essentially all human infections with VEEV are symptomatic,[83,84] only a small percentage manifest neurological involvement.[155] In one epidemic, the ratio of encephalitis to infections was estimated at less than 0.5% in adults, although possibly as high as 4% in children.[156] Mild CNS involvement is evidenced by lethargy, somnolence, or mild confusion, with or without nuchal rigidity.[8] Seizures, ataxia, paralysis, or coma indicate more severe CNS involvement. In children with overt encephalitis, case fatalities may be as high as 35% compared with 10% for adults.[157] However, for those adults who survive encephalitic involvement, neurological recovery is usually complete,[158] although one report documented motor disorders and an increased incidence of seizures in children following VEE outbreaks.[158] Abortions and increased fetal deaths have also been attributed to VEEV infection.[27,152] School-aged children are believed to be more susceptible to a fulminant form of disease, which follows a lethal course over 48 to 72 hours in which depletion of lymphoid tissues is prominent.[120,159,160]

In the first 3 days of illness, leukopenia and elevated aspartate aminotransaminase are common. For those with CNS involvement, a lymphocytic pleocytosis of up to 500 cells per microliter can be observed in the cerebrospinal fluid (CSF). The CSF pleocytosis may be acutely polymorphonuclear but soon becomes predominantly lymphocytic.

Specific diagnosis of VEEV infection can be accomplished by virus isolation, serologic testing, identification of virus-specific nucleic acid, or all three.[59,161–165] Identification of the VEEV subtype of an isolate involved can usually be determined by cross-neutralization tests or more reliably by genetic sequence analysis. During the first to third days of symptoms of nonspecific febrile illness, VEEV may be recovered from either the serum or the nasopharynx.[166] Despite the theoretical possibility of person-to-person transmission of virus present in the nasopharynx, no such occurrences have been reported. In NHPs, the virus is found in the blood for the first 2 to 3 days after aerosol exposure but levels are low compared to what has been reported for natural infection and may not be detectable after fever onset for enzootic strains.[131,153] VEEV can be isolated from the nasopharynx of NHPs for up to 5 days postexposure after aerosol exposure of naïve animals. Antibodies detectable by an HI assay, enzyme-linked immunosorbent assay, or plaque-reduction neutralization tests appear as viremia diminishes. Complement-fixing antibodies make their appearance later during convalescence. VEEV immunoglobulin M (IgM) antibodies are present in acute phase sera,[84] and VEEV IgM tests do not react with sera from patients with EEEV or WEEV.[167] Since patients with encephalitis typically come to evaluation later in the course of clinical illness, virus is recovered less often from them,[131] and they usually have virus-specific serum antibody by the time of clinical presentation.[168] Immunity after infection is probably lifelong to the homologous serotype, but cross-immunity may be weak or nonexistent to heterologous serotypes.[37,61,62] Thus, when viewed either as an endemic disease threat or as a potential biological warfare threat, adequate immunization may require polyvalent vaccines.

Eastern Equine Encephalitis

In North America, EEEV is maintained in a natural transmission cycle between *Culiseta melanura* mosquitoes and passerine birds in freshwater hardwood swamps and forested areas, primarily in the Atlantic and Gulf Coast states. EEEV outbreaks are typically recognized when severe equine or human encephalitis occurs near such areas.[169] In the southeastern United States, *Culex erraticus* may play an important role in the transmission of the virus to humans and horses.[170–172] During vectorborne EEEV epidemics, the incidence of human infection is low (<3% of the population at risk), and the neurological attack rate in one outbreak was estimated at 1 in every 23 cases of human infection.[173] However, the effect on morbidity and mortality of aerosol-acquired (the expected route of infection in a biological warfare offensive) EEEV infection in humans is unknown, although animal studies suggest that EEEV by aerosol is lethal.[145] The incubation period in humans varies from 5 to 15 days. Adults typically exhibit a febrile prodrome for up to 11 days before the onset of neurological disease.[174] However, illness in children exhibits a more sudden onset.[175] In natural outbreaks, viremia occurs during the febrile prodrome,[176] but is usually undetectable by the time clinical encephalitis develops, when HI and neutralizing antibodies become evident.[173] Despite the development of a prompt and neutralizing humoral response, the virus is not eliminated from the CNS, and progressive neuronal destruction and inflammation continue.

EEE is the most severe of the arboviral encephalitides, with high mortality and severe neurological sequelae.[177] During EEEV outbreaks, the attack, morbidity, and fatality rates are highest in young children[178] and elderly people.[179] Overall, approximately 4% to 5% of human EEEV infections result in clinically apparent EEE in the United States, with an average of six human cases of EEE reported annually.[180] Case fatality rates are estimated to be from 30% to 70%, but asymptomatic infections and milder clinical illness are underreported. The illness is characterized by a nonspecific prodrome followed by severe headache, high fevers, lethargy, and seizures, often with rapid progression to coma and death.[181–183] Motor involvement with paresis is common during the acute phase of the illness. Major disturbances of autonomic function, such as impaired respiratory regulation or excess salivation, may dominate the clinical picture. Between 30% and 70% of survivors are left with long-term neurological sequelae such as seizures, spastic paralysis, and cranial neuropathies. Cognitive impairment ranges from minimal brain dysfunction to severe dementia.

In a recent retrospective study of 15 cases of EEE in children, fever, headache, and seizures were the most common clinical signs, with 87% of the patients becoming stuporous or comatose during the first 3 days of hospitalization.[184] Radiographic lesions were noted in the basal ganglia, thalami, and cerebral cortex. This study found an important association between a short prodrome (ie, the time between initial nonspecific symptoms and the first major neurologic symptom) and an increased risk for death or for severe disease. The eight patients who had a poor outcome all had a prodrome of 2 days or less, and all four deaths occurred in this group.[184]

Clinical laboratory findings in patients with EEE often demonstrate an early leukopenia followed by a leukocytosis. Elevated opening pressure is commonly noted on lumbar puncture and, especially in children, the CSF lymphocytic pleocytosis may reach a cell count of thousands of mononuclear cells per microliter. However, in a recent report, neutrophilic pleocytosis with elevated levels of protein were the most consistent findings when CSF was evaluated within the first week of symptom onset.[184] Specific diagnosis of EEE depends on virus isolation or serologic testing in which rising titers of HI, complement-fixing, or neutralizing antibodies are observed. IgM antibodies are usually detectable in acute-phase sera.[167] As with other alphaviruses, neutralization tests are the most specific. Immunohistochemistry can also be performed postmortem on fixed brain samples.[145] In NHPs exposed by aerosol to EEEV, the period from fever onset until the animal is moribund is less than 48 hours regardless of dose.[145]

Western Equine Encephalitis

Like VEEV, naturally acquired WEEV (by mosquito bite) is less virulent for adult humans than for equids and children, with lower rates of fatalities and neurological sequelae.[185] As with EEEV, infants and elderly people are especially susceptible to severe clinical illness and neurological sequelae, with case fatality rates of about 10%. Highlands J virus, an antigenically related member of the WEE complex that is isolated frequently in the eastern United States, rarely infects humans.

The incubation period is 5 to 10 days for natural WEEV infection. In NHPs infected by aerosol, the incubation period is 4 to 5 days.[149] A large percentage of patients with vectorborne infections are either asymptomatic or present with a nonspecific febrile illness or aseptic meningitis. The ratio of encephalitis cases per infection has been estimated to vary from 1 per 1,150 in adults, to 1 per 58 in children, to 1 per 1 in infants.[74] However, the severity of the syndrome and the incidence of inapparent infection almost certainly depend on the strain and dose of the virus, and the route of infection. Some unusual isolates show high virulence in laboratory animals[138,186,187] and in one study of laboratory-acquired infections in adults, two of five patients died.[35] Symptoms usually begin with malaise, headache, and fever, followed by nausea and vomiting.[188] Telemetry data from NHPs exposed to WEEV by aerosol revealed, in addition to fever, increases in heart rate and changes in electrocardiograph readings, indicative of sinus tachycardia.[189] A transient leukopenia followed by a pronounced leukocytosis composed almost entirely of segmented neutrophils correlated with a poor prognosis. Fever severity also correlated with a poor prognosis. Over the next few days the symptoms intensified, and in some cases, somnolence or delirium progressed into coma. The severity of neurological involvement is inversely related to age, with more than 90% of children younger than 1 year exhibiting focal or generalized seizures.[190] Physical examination typically reveals nuchal rigidity, impaired sensorium, and upper motor neuron deficits with pathologically abnormal reflexes.

Patients with the most severe disease usually die within the first week of clinical illness, with case fatalities averaging 10%. Other patients begin a gradual convalescence after the first week of encephalitic symptoms. Most adults recover completely, but it may take months to years to recuperate from fatigability, recurrent headaches, emotional lability, and impaired concentration.[191] Some patients have permanent residua of motor weakness, cognitive deficits, or a seizure disorder. Children carry a higher incidence of

neurological sequelae, ranging from less than 1% in those older than 1 year, to 10% in infants 2 to 3 months old, to more than 50% in newborns. Congenital infection in the last trimester of pregnancy has been described, with resultant encephalitis in the infants.[192] Laboratory accidents involving aerosol exposure to WEEV have been documented and the mortality of those limited cases was 40%.[35] In NHPs, aerosol exposure to a dose equivalent to 10 times the median infective dose produced fever, and 50% of the animals developed clinical signs indicative of encephalitis. Twenty-five percent of those animals died from the infection by day 9 postexposure.[189]

Viremia is rarely detectable by the time patients present with encephalitic symptoms, but IgM, HI, and neutralizing antibodies are generally detected by the end of the first week of illness, and they increase in titer during the following week.[167,193,194] In NHPs exposed to aerosolized WEEV, the virus was not detectable in the serum or nasopharynx postexposure.[189] However, low levels of virus were detected in CSF. Antibody responses were not detectable by an enzyme-linked immunosorbent assay or plaque-reduction neutralization test until day 9 postexposure in survivors, which was after control animals had died from the infection. Complement-fixing serologic responses generally appear in the second week and rise thereafter. Isolation of virus with up to 4-fold increase in titer is diagnostic, but because of serologic cross-reactions with other alphaviruses, neutralization tests are preferred. Examination of the CSF reveals a lymphocytic pleocytosis ranging from 10 to 400 mononuclear cells per microliter. WEEV may occasionally be isolated from the CSF taken within the first 2 days of fever, and it is frequently recovered from brain tissue on postmortem examination.[195] Survival from natural infection presumably confers long-term immunity; however, it may not protect against aerosol exposure.[196]

Differential Diagnosis of Alphavirus Encephalitis

Most acute infections with VEEV and WEEV produce a moderately severe but nonspecific clinical illness, consisting of fever, headache, and myalgias. Therefore, in a potential biological warfare scenario, alphaviruses should be considered in the differential diagnosis whenever epidemic febrile illness occurs, especially if several patients progress to neurological disease. Sick or dying equids near an epidemic febrile illness among troops should immediately suggest the possibility of large-scale alphavirus exposure. Other potential biowarfare agents that may infrequently produce or imitate a meningoencephalitic syndrome include Rift Valley fever virus, *Brucella* species, *Yersinia pestis*, *Salmonella typhi*, *Coxiella burnetii*, and botulinum toxin. As with any meningoencephalitis diagnosis, it is imperative to rule out any potential cause that may be specifically treatable.

For encephalitis cases that are more sporadic in their occurrence, other important viral etiologies that might not be readily discriminated from the alphaviruses by

TABLE 20-3

SOME IMPORTANT VIRAL CAUSES* OF ENDEMIC ENCEPHALOMYELITIS

Virus Family	Genus	Species
Togaviridae	*Alphavirus*	Eastern equine encephalitis virus
		Western equine encephalitis virus
		Venezuelan equine encephalitis virus
Flaviviridae		St Louis encephalitis virus
		Murray Valley encephalitis virus
		West Nile virus
		Japanese encephalitis virus
		Dengue virus
		Tickborne complex viruses
Bunyaviridae		LaCrosse virus
		Rift Valley fever virus
		Toscana virus
Paramyxoviridae	*Paramyxovirus*	Mumps virus
	Morbillivirus	Measles virus
	Henipavirus	Hendra virus
		Nipah virus
Arenaviridae	*Arenavirus*	Lymphocytic choriomeningitis virus
		Machupo virus
		Junin virus
		Guanarito virus
Picornaviridae	*Enterovirus*	Poliovirus
		Coxsackievirus
		Echovirus
Reoviridae		Colorado tick fever virus
Rhabdoviridae	*Lyssavirus*	Australian bat lyssavirus
		Rabies virus
Herpesviridae	*Herpesvirus*	Herpes simplex virus types 1 and 2
		Epstein-Barr virus
		Cytomegalovirus
Adenoviridae	*Adenovirus*	

*Not all-inclusive.

EXHIBIT 20-1

NONVIRAL CAUSES OF ENCEPHALOMYELITIS

Treatable infectious conditions that can mimic viral encephalitis:
- Partially treated bacterial meningitis
- Brain abscess
- Subdural empyema
- Embolic encephalitis associated with bacterial endocarditis
- Lyme disease
- Tuberculous meningitis
- Fungal meningitis
- Rocky Mountain spotted fever
- Cat scratch disease
- Cerebral malaria
- Trypanosomiasis
- Toxoplasmosis

Vascular, autoimmune, and neoplastic diseases that can mimic infectious meningoencephalitis:
- Lupus cerebritis
- Cerebral and granulomatous arteritis
- Lymphomatous cerebritis
- Whipple's disease
- Behçet syndrome
- Carcinomatosis meningitis

clinical features are listed in Table 20-3. This list is not all-inclusive but suggests other viral encephalitides that should be considered if a patient presents, a priori, with an encephalitic syndrome.

Epidemiological, historical, and laboratory information remains critical to differential diagnosis. Immediate and careful consideration must be given to treatable infections that may mimic viral encephalitis (Exhibit 20-1) because prompt and appropriate intervention can be lifesaving. In addition, vascular, autoimmune, and neoplastic diseases may imitate infectious meningoencephalitis.

For endemic meningoencephalitic disease that occurs outside biowarfare theaters, the geographical locale and the patient's travel history are of preeminent importance in diagnosing an arboviral encephalitis. Risk for disease is increased relative to the patient's amount of arthropod contact near swampy or forested areas during the summer in temperate climates or year-round in the tropics. Encephalitic illness of equids in the surrounding locale is an important indication of ongoing transmission of encephalitic alphaviruses. Animal studies have indicated that the virus may not be detectable in the serum during the febrile period, and antibody responses may be weak or nonexistent, making diagnosis difficult, which is particularly true for WEEV. Examination of the CSF, including viral cultures or reverse transcription polymerase chain reaction, is critical in differentiating bacterial from viral infections, and infectious from noninfectious etiologies. Serum and CSF tests based on antibody or genetic detection hold great promise in more rapid diagnosis of infectious encephalitis. In some instances it will be necessary to (*a*) institute therapy for possible, treatable, infecting organisms and (*b*) await definitive laboratory diagnostic tests.

Medical Management and Prevention

No licensed vaccines or therapeutics currently exist for the alphaviral encephalitides; therefore, treatment is aimed at management of specific symptoms (eg, anticonvulsant medication and airway protection). The high fever occasionally produced by WEEV infection in humans is a special problem among the arboviral encephalitides that may require aggressive antihyperthermia measures.[194,197] The US Army has extensive experience with IND live-attenuated and formalin-inactivated vaccines in humans (which are discussed later in this chapter).

Use of an effective vaccine in horses would also prevent outbreaks of epizootic VEE, as equines are the major amplifying species for VEEV. However, vaccination of horses is not a useful public health tool for EEEV, WEEV, or enzootic VEEV, because horses are not important as amplifying hosts for these viruses. Integrated mosquito control measures can also have significant impact on ameliorating epidemic transmission.

IMMUNOPROPHYLAXIS

Relevant Immune Effector Mechanisms

The equine encephalitis viruses constitute both an endemic disease threat as well as a biological warfare threat; therefore, adequate immunoprophylaxis of military and civilian personnel will require protection against both vectorborne and aerosol-acquired infections. The requirements for protection against parenteral infection are well described, but the requirements for protection against infectious aerosols are more stringent and remain largely unidentified. Within a few days of infection with an alphavirus, specific antibodies can be detected in the serum of animals or humans. Within 7 to 14 days, a virus-neutralizing

antibody response develops, as measured by the ability of serum antibodies to block virus infectivity in vitro or in vivo. Protection from mosquito-vectored alphavirus disease is believed to be primarily mediated by this virus-specific neutralizing antibody response, which is largely directed against epitopes on the E2 glycoprotein.[198,199] Protection mediated by nonneutralizing antibodies to alphaviruses, directed largely at epitopes on the E1 glycoprotein, has also been described.[196,200] Protection from aerosol exposure correlated with serum neutralization or antibody titers in some studies in mice, hamsters, and NHPs,[130,131,135,198,201] but this is not consistently the case.

There have also been reports of virus-specific cytotoxic T cell responses induced against alphaviruses.[202–204] Although cytotoxic T cell activity was not detected in early studies with a VEEV vaccine in mice,[205] more recent studies have demonstrated a role for certain subsets of T cells in protection against VEEV.[206–208]

Nonspecific immune responses that occur following alphavirus infection include the induction of secretion of interferon (IFN)[209–213] and the activation of cytotoxic macrophages.[214] Several studies have demonstrated the importance of the innate immune response, specifically IFN-α, in resistance to alphavirus infection. Studies with Semliki Forest virus and VEEV have shown that IFNα/β receptor knockout mice are more susceptible to infection.[215–217] Pre- and postexposure administration of IFN or inducers of interferon in vivo may be effective for protection against alphaviruses.[218,219] IFN-β was beneficial in protection against the Semliki Forest virus peripheral challenge when administered up to 6 days postexposure. Mice were resistant to subcutaneous challenge with VEEV TrD and partially protected from inhalation challenge when administered pegylated IFN-α on days -2 and +5 relative to exposure.[220] Pretreating mice with polyinosinic:polycytidylic acid (poly I:C) afforded partial protection against peripheral challenge with EEEV,[215] and poly I:C with added carboxymethylcellulose and poly-L-lysine similarly induces protection against respiratory challenge with WEEV.[219] Although these studies clearly indicate the importance of interferons in host resistance to alphavirus infections, further study is necessary to determine the efficacy of IFN-α for prophylactic or therapeutic use in humans.

Passive Immunization

Passive transfer of neutralizing antisera or monoclonal antibodies to naive recipients protects animals from subsequent parenteral challenge with homologous VEEV strains.[201,212,221] Passive transfer of nonneutralizing, anti-E1 monoclonal antibodies directed against appropriate epitopes is also protective against SINV,[200] WEEV,[198] and VEEV.[201] Monoclonal antibodies specific for the E3 protein of VEEV IAB do not neutralize VEEV IAB TrD in vitro; however, they inhibit VEEV IAB TrD production in infected cells and protect against intraperitoneal challenge with VEEV IAB TrD after passive transfer in mice.[106] In contrast, for the respiratory route of infection, uniform protection was not observed after passive transfer of hyperimmune serum to hamsters[198] neutralizing monoclonal antibodies to mice,[222] suggesting that either additional immune mechanisms or the presence of protective antibodies along the respiratory tract may be needed. The time between the administration of immune serum and virus exposure may also be relevant. Protection of mice from intracerebral inoculation with WEEV was observed if immune serum was given no more than 3 days before virus exposure.[223,224] Similarly, monkeys passively immunized with horse antiserum to EEE or WEE resisted intranasal challenge from homologous virus 24 hours later, but they were unable to resist a second challenge with the same virus 7 weeks later.[225] However, as the immune serum given in both studies was xenogeneic, the loss of protective capacity was presumably related in part to active clearance of the immune serum by the recipients.

The effect of administering immune serum to animals after the establishment of intracerebral infections has also been evaluated. Several studies using different alphaviruses demonstrated at least partial protection if the immune serum was administered within 24 hours of infection.[223–228] Other researchers have suggested that postinfection serum transfer may also cause a more severe pathology, or may merely delay the onset of disease symptoms.[41] Aggressive serotherapy following infections of two laboratory workers who developed acute WEE encephalitis resulted in the survival of one patient,[39] but was ineffective in the second patient.[225]

In an EEE outbreak in New Jersey in 1959, 22 of 32 diagnosed patients died. Most patients had demonstrable antibody during the onset or progression of encephalitis, and neutralizing antibody titers in sera from patients who died were generally similar to those observed in patients who recovered.[229] This finding, coupled with animal studies indicating that transfer of virus-neutralizing anti-sera was unable to prevent progression of disease if infection of the brain was firmly established as described above, suggests that serotherapy would be an ineffective means of treatment for these virus infections unless initiated early in the course of disease.

TABLE 20-4

VACCINES AVAILABLE FOR VEE, EEE, AND WEE VIRUSES

Vaccine	Form/Strain	Dose (mL)/ Route of Administration	Responding Schedule	Booster Dose/%	Duration*	Route
VEE (TC-83) Attenuated	TrD	0.5 mL/sc	Day 0	82%	92%	C-84/sc
VEE (C-84)†	Inactivated TC-83	0.5 mL/sc	After TC-83	76% NR‡ 100% WT§	60% 100%	0.5 mL/sc
EEE	Inactivated PE-6¥	0.5 mL/sc	Days 0, 28	58%	75%	0.1 mL/id
WEE	Inactivated CM-4884¥	0.5 mL/sc	Days 0, 7, 28	50%	20%	0.5 mL/sc

*% of responders whose virus-neutralizing titers persist for at least 1 year
†current IND protocols specify use of C-84 only as a booster vaccine
‡TC-83 nonresponders
§TC-83 responders given C-84 to boost waning titers
¥laboratory designation
EEE: Eastern equine encephalitis
id: intradermal
IND: investigational new drug
sc: subcutaneous
TC: cell culture
TrD: Trinidad donkey
VEE: Venezuelan equine encephalitis
WEE: Western equine encephalitis

Active Immunization

Investigational New Drug Vaccines

Although no vaccines exist against the encephalitic alphaviruses that are licensed for use in humans, the US Army has developed vaccines that are currently used under IND status to protect at-risk personnel including the live-attenuated VEEV vaccine TC-83 and inactivated vaccines for VEEV (C-84), EEEV, and WEEV. The characteristics of these vaccines and the responses induced in human vaccinees are summarized in Table 20-4.

Live Vaccines

The TC-83 VEEV vaccine, which was developed in 1961 by serial passage of the virulent TrD strain in fetal guinea pig heart cells,[230] is administered subcutaneously at 1×10^4 to 2×10^4 plaque-forming units per 0.5 mL dose. The vaccine was used initially in laboratory and field personnel at risk for exposure to VEEV,[231] and more than 6,000 people received the vaccine between 1964 and 1972.[231] For reasons that remain unclear, approximately 20% of the people who receive TC-83 fail to develop a detectable neutralizing antibody response and presumably would not be protected if exposed to the virus. Another 20% to 25% of vaccine recipients experience clinical reactions ranging from mild transient symptoms to fever, chills, sore throat, and malaise in some cases sufficient to require bedrest.[230,232] However, for recipients who respond with postvaccination titers of at least 1 per 20, long-term follow-up studies have shown that titers persist for several years.[233] In humans, documented vaccine-breakthrough infections have been attributed largely to exposure to heterologous, enzootic strains of VEEV.[37,61,62] Although pregnant mares were not adversely affected by TC-83,[234] pregnant women are advised not to receive the TC-83 vaccine because wild-type VEEV may have been associated with spontaneous abortions or stillbirths during epidemics in Venezuela in 1962 and 1995.[27,121]

In animals, TC-83 vaccination protects hamsters from a lethal VEEV subcutaneous or aerosol challenge,[135] although up to 20% of hamsters may die from reactions to the vaccine.[127,235] Subcutaneous immunization of monkeys with the vaccine produces neutralizing antibody responses and protection from virulent VEEV delivered by peripheral or intranasal challenge.[134] However, TC-83 provides only partial protection against aerosol challenge in outbred mice.[139] TC-83 has been extensively administered to horses, burros, and mules, in part because large numbers of equids were vaccinated during the 1969–1970 and 1995 epizootics.[236,237] TC-83 immunization produces febrile

responses and leukopenia in some equids,[238,239] but neutralizing antibody responses to homologous (serotype IAB) virus eventually develop in 90% of these animals.[238,240] Although it was difficult to accurately assess vaccine efficacy under the conditions of an ongoing epizootic, herds of animals known to have been immunized at least 2 weeks before any disease occurrence in the area did not sustain any VEEV-related deaths, whereas unimmunized herds experienced up to 60% mortality rates.[232]

The phenomenon of vaccine interference, in which prior immunity to heterologous alphaviruses inhibits vaccine virus replication and subsequent immune responses, is an unresolved problem with the use of TC-83 and presumably with other live-attenuated alphavirus vaccines. This occurrence has been observed in horses,[241,242] in which preexisting antibodies to EEE and WEE may have interfered with TC-83 vaccination. Interference has also been observed in humans, in which preexisting immunity to a live alphavirus vaccine inhibited effective subsequent immunization with a second, different alphavirus vaccine.[243] However, a recent study found no evidence for interference when vaccines for VEE, WEE, and EEE were administered simultaneously to NHPs. Interference may greatly depend on the nature of the vaccine and the virus strain(s) selected for both the vaccine and in vitro neutralization studies.[244]

Inactivated Vaccines

Early attempts to develop an inactivated VEEV vaccine resulted in preparations that contained residual live virus and caused disease in 4% of those who received it.[226,245] Development of a formalin inactivated TC-83 VEEV vaccine (C-84) was initiated because of the problems associated with incomplete inactivation.[246] Initial clinical trials with the C-84 inactivated vaccine were begun in 1976 in 14 volunteers previously immunized with TC-83, and subsequently in 14 naive volunteers.[247] The vaccine was found to be safe and elicited only mild tenderness at the injection site. Although C-84 was immunogenic, three doses were required to maintain detectable neutralizing antibody titers in recipients. A subsequent study has shown that most of the TC-83 nonresponders and all of the individuals with waning titers responded to a booster dose of C-84 with a high probability of maintaining a titer for 3 years.[231] However, the observation that hamsters given C-84 vaccine were protected from subcutaneous challenge but not from an aerosol exposure to VEE virus[135] raised concerns that C-84 vaccination may not protect at-risk laboratory workers from aerosol exposure. Therefore, C-84 is currently administered only as a booster immunogen.

The PE-6 strain of EEEV was passed in primary chick-embryo cell cultures, and then it was formalin treated and lyophilized to produce an inactivated vaccine for EEEV.[248] This vaccine is administered as a 0.5-mL dose subcutaneously on days 0 and 28, with 0.1-mL intradermal booster doses given as needed to maintain detectable neutralizing antibody titers. In initial clinical trials, only mild reactions to the vaccine were observed, and immunogenicity was demonstrated.[249] The vaccine was given to 896 at-risk laboratory workers between 1976 and 1991 with no significant clinical reactions observed. A long-term follow-up study of 573 recipients indicated a 58% response rate after the primary series, and a 25% chance of failing to maintain adequate titers for 1 year. Response rates and persistence of titers increased with the administration of additional booster doses.[231]

A formalin inactivated WEEV vaccine was similarly prepared using the B-11 or CM-4884 virus strain, and it caused only mild clinical reactions when administered to WEEV-naive individuals, according to Phillip Pittman, when he was the former chief of the special immunization program at the US Army Medical Research Institute of Infectious Diseases in 1996. Between 1976 and 1990, 359 laboratory workers were immunized with this vaccine. Long-term follow-up studies have indicated that administration of three 0.5 mL doses subcutaneously on days 0, 7, and 28 results in a 50% response rate (neutralization titer >1:40) after the primary series. Only 20% of the recipients maintain a titer for 1 year, although this level can be increased to 60% to 70% with additional booster immunizations, according to Pittman.

As with the live-attenuated alphavirus vaccines, immune interference has also been observed after vaccinations with the inactivated alphavirus vaccines. Volunteers who received the inactivated EEEV and WEEV vaccines before receiving the live-attenuated VEEV vaccine had significantly lower rates of neutralizing antibody response than those receiving the VEEV vaccine before the EEEV and WEEV vaccines.[250]

Next Generation Alphavirus Vaccines

Significant limitations are associated with the live-attenuated VEEV and formalin-inactivated VEEV, EEEV, and WEEV IND vaccines used to protect at-risk personnel. These limitations include the reactogenicity of the live-attenuated vaccine, the poor immunogenicity of the formalin-inactivated vaccines, and the demonstrated immune interference issues associated with these vaccines. As a result, efforts are underway using many different platforms to develop next-generation vaccines that can safely and

effectively protect against VEEV, EEEV, and WEEV; next-generation subunit, live-attenuated, inactivated, DNA, virus replicon particle, and SINV-based chimeric vaccines are all at various stages of development. Subunit vaccines consisting of glycoproteins produced in *Escherichia coli* or baculovirus expression systems have provided limited success in mouse models.[251–254] Inactivated virus vaccines provided efficacy against aerosol challenge in mice.[255] However, further study is required to determine the efficacy of subunit and inactivated virus vaccines in NHPs. SINV-based chimeric virus vaccines are immunogenic and protect mice against VEEV, EEEV, and WEEV.[256–260] A recent study demonstrated a chimeric SINV–EEEV vaccine candidate protected most NHPs (82%) from lethal EEE disease following aerosol infection.[261] The US Army has extensive experience in the development of next-generation live-attenuated, DNA, and virus-like replicon particle vaccines to protect against the encephalitic alphaviruses.

V3526

The next-generation live-attenuated VEEV vaccine, V3526, was created by mutation of the furin cleavage site of PE2 in wild-type VEEV IAB combined with a second-site suppressor mutation in the E1 protein.[262] These mutations significantly reduced the neurovirulence of V3526 as compared to the parent clone and TC-83 in mice, NHPs, and horses, and they stabilized the attenuated phenotype.[130,150,151,263,264] V3526 has been shown to effectively elicit protective immune responses in rodents, NHPs, and horses against lethal subcutaneous or aerosol challenges with VEEV IAB TrD as well as other VEEV subtypes and related viruses (IC, IE, and Mucambo virus).[135,130,150,151,263] Mice were protected from aerosol challenge with VEEV IAB TrD for up to 1 year following vaccination with V3526.[151] Furthermore, immune interference was not observed when V3526 was administered to mice previously vaccinated with other alphavirus vaccines. Based on the success of V3526 in nonclinical studies, a phase 1 clinical trial was conducted to evaluate the safety, tolerability, and immunogenicity of this vaccine candidate in humans. V3526 was immunogenic in virtually all recipients, with robust immune responses elicited after administration of a single dose of the vaccine down to doses as low as 25 plaque-forming units. However, a significant number of the vaccinated subjects experienced adverse events consistent with a viral syndrome to include headache, fever, malaise, myalgia, and sore throat.[265] Based on these findings, clinical development of V3526 was discontinued. Gamma irradiated and formalin-inactivated V3256 vaccines have subsequently been tested in mice, but these have not progressed beyond animal studies.[266–268]

DNA Vaccines

DNA vaccination with plasmids that express protein antigens within cells has numerous inherent advantages as a platform for the development of next-generation vaccines. Some of these benefits include that DNA vaccines:

- can be rapidly produced using well-established Good Manufacturing Practices and without the need to propagate a pathogen or inactivate an infectious organism;
- avoid problems of preexisting immunity resulting from a lack of a host immune response to the vector backbone; and
- have been demonstrated to be safe in numerous human clinical trials.[269]

Although a DNA vaccine expressing the structural proteins (C-E3-E2-6K-E1) of VEEV IAB TrD from the wild-type genes delivered by particle-mediated epidermal delivery or "gene gun" elicited strong overall antibody responses in multiple animal species, the neutralizing antibody responses were low and only partial protection against VEEV IAB TrD aerosol challenge was observed in mice and NHPs.[270–272] A codon-optimized DNA vaccine construct expressing the structural proteins of VEEV IAB TrD minus the capsid protein delivered by intramuscular electroporation elicited improved antibody responses, including high levels of neutralizing antibodies in multiple animal species, and it provided protective immunity against VEEV IAB TrD aerosol challenge in mice and NHPs.[273] Based on these results, a phase 1 clinical trial to evaluate the safety, tolerability, and immunogenicity of this vaccine candidate in humans has been initiated. A trivalent formulation of VEEV, EEEV, and WEEV DNA vaccine constructs has also been extensively evaluated in animals to reach the goal of developing a vaccine capable of simultaneously eliciting protective immunity against VEEV, EEEV, and WEEV. Important to this goal, the immunogenicity of the combined VEEV, EEEV, and WEEV DNA vaccines was not significantly reduced as compared to the individual DNA vaccines, and protection against VEEV, EEEV, and WEEV aerosol challenge has been observed in mice and NHPs. As a result, nonclinical studies required to advance this trivalent DNA vaccine formulation into phase 1 clinical testing in humans are being conducted.

Virus Replicon Particle Vaccines

Alphavirus-based replicon systems, derived by deletion of the genes encoding the viral structural proteins from full-length genomic complementary DNA clones and replacing these with heterologous genes of interest, represent a promising method for the development of next-generation vaccines.[274] Virus-like replicon particles (VRPs) are produced in vitro following cotransfection of cells with the replicon RNA, which express the nonstructural proteins in cis and helper RNAs, which supply the structural proteins in trans.[275] The immunogenicity and protective efficacy of VRPs expressing VEEV, EEEV, or WEEV envelope glycoprotein genes containing the furin cleavage site mutation in PE2 have been extensively evaluated in mice and NHPs.[244] The VEEV, EEEV, and WEEV VRP vaccines elicited strong neutralizing antibody responses when administered individually and in combination to mice. In addition, mice receiving the individual or combined VRP vaccines were protected from respective VEEV, EEEV, or WEEV aerosol challenge up to 12 months after vaccination. NHPs receiving the individual VEEV or EEEV or the combined VRP vaccines developed strong neutralizing antibody responses and were protected against VEEV and EEEV aerosol challenge, respectively. However, the individual WEEV and combined VRP vaccines elicited low or no neutralizing antibodies against WEEV in NHPs, and incomplete protection against WEEV aerosol challenge was observed. The VEEV, EEEV, and WEEV VRP vaccines have not yet progressed beyond nonclinical studies.

THERAPEUTICS

No licensed therapeutics are available for the specific treatment of alphavirus infections in humans. However, several studies have reported the identification of compounds with in vitro efficacy against alphaviruses.[276–280] In three of these studies, the identified compounds targeted proteins of the viral polymerase complex.[276–278] Two other studies identified compounds targeting host protein kinases.[279,280] The efficacy of several of these compounds was demonstrated in rodent models of VEEV or WEEV infection[276–279]; however, no studies have reported efficacy of any compounds in NHP models of alphavirus infection.

SUMMARY

The equine encephalitis viruses consist of three antigenically related viruses within the *Alphavirus* genus of the family *Togaviridae*: VEEV, WEEV, and EEEV. These viruses are vectored in nature by various species of mosquitoes and cause periodic epizootics among equines. Infection of equines with virulent strains of any these viruses produces a similar clinical course of severe encephalitis with high mortality. However, the clinical course following infection of humans differs. EEE is the most severe of the arbovirus encephalitides, with case fatality rates of 50% to 70%. WEEV is generally less virulent for adults, but the infection commonly produces severe encephalitis in children, with case fatality rates approaching 10%. In contrast, encephalitis is rare following VEEV infection, but essentially all infected individuals develop a prostrating syndrome of high fever, headache, malaise, and prolonged convalescence.

Although natural infections are acquired by mosquito bite, these viruses are also highly infectious in low doses as aerosols. These viruses can be produced in large quantities using inexpensive and unsophisticated systems, are relatively stable, and are readily amenable to genetic manipulation. For these reasons, the equine encephalomyelitis viruses are considered credible biological warfare threats.

No specific therapy exists for infections caused by these viruses. A live-attenuated vaccine for VEEV (TC-83) and inactivated vaccines for VEEV, EEEV, and WEEV have been developed and are used under IND status. Although these vaccines are useful in protecting at-risk individuals, they have certain disadvantages, and improved vaccines are being developed.

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Chapter 21

HEMORRHAGIC FEVER-CAUSING MAMMARENAVIRUSES

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SUMMARY

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INTRODUCTION

The family *Arenaviridae* includes the two genera *Mammarenavirus* and *Reptarenavirus*, which are established to accommodate mammalian and reptilian arenaviruses, respectively. Seven mammarenaviruses cause viral hemorrhagic fever in humans: Lassa virus (LASV), Lujo virus (LUJV), Chapare virus (CHAPV), Guanarito virus (GTOV), Junín virus (JUNV), Machupo virus (MACV), and Sabiá virus (SABV). The clinical course and pathology of the viral hemorrhagic fevers caused by these viruses can differ, and therefore various diagnosis and treatment options are available. This chapter summarizes similarities and disparities between the viruses and the diseases they cause.

HISTORY AND EPIDEMIOLOGY

Old World Mammarenaviruses

Lassa fever is a severe disease common in areas of western sub-Saharan Africa (Nigeria, Liberia, Guinea, Sierra Leone; Figure 21-1). Lassa fever was first described in Jos, Nigeria, in 1969 in a hospitalized patient and a caretaker, both of whom became severely ill and subsequently died.[1] The etiologic agent of Lassa fever is LASV. The case fatality rate of Lassa fever is about 1% to 2% in the endemic areas, with an estimated 300,000 to 500,000 infections annually. The disease is especially severe late in pregnancy.[2] Infections tend to be more common in February to April compared to the rest of the year.

In September and October of 2008, LUJV was discovered during a nosocomial viral hemorrhagic fever outbreak. The index case became infected in Lusaka, Zambia (Figure 21-1), but the origin of

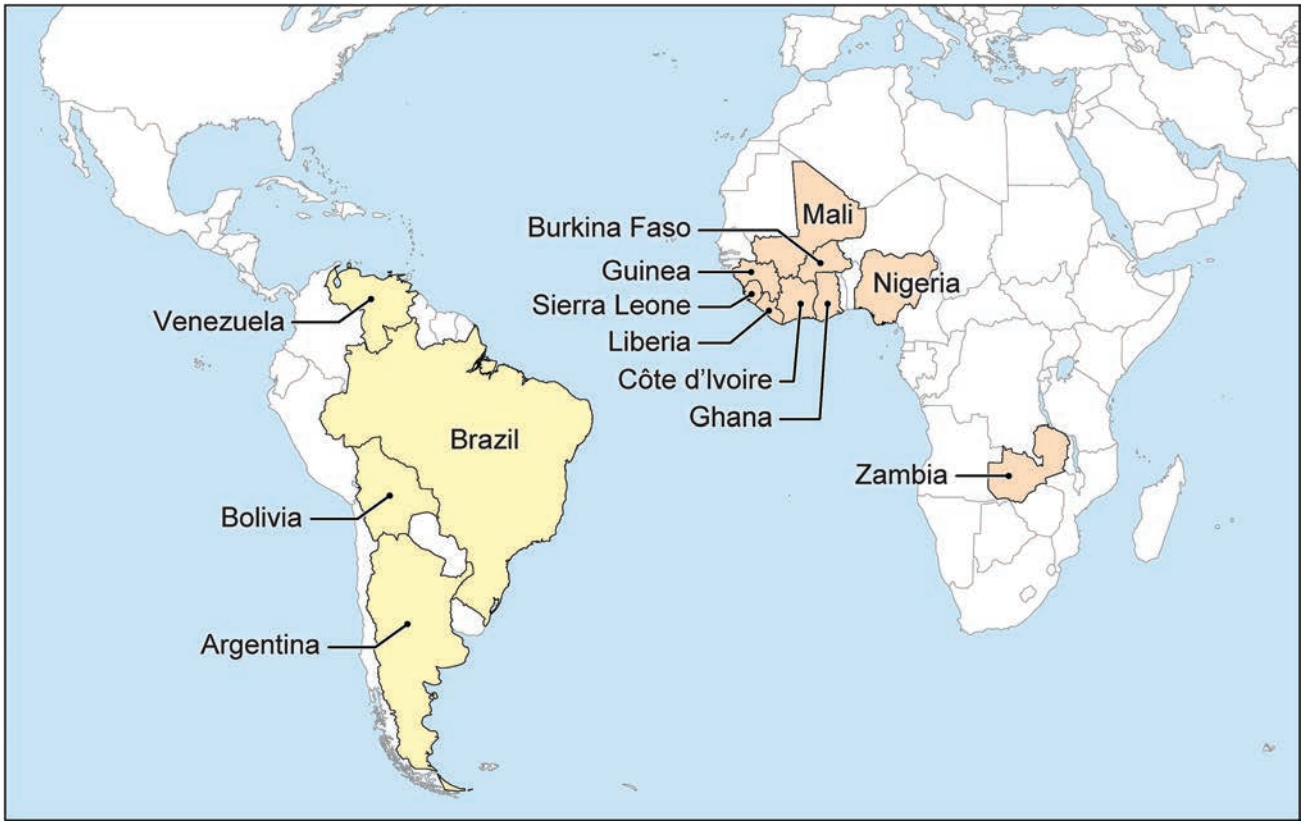


Figure 21-1. Global distribution of viral hemorrhagic fever-causing mammarenaviruses. Mammarenaviruses are phylogenetically and serologically differentiated into Old World (*orange*) and New World (*yellow*) groups. Old World mammarenaviruses: Lujo virus (Zambia) and Lassa virus (other highlighted countries). New World mammarenaviruses: Guanarito virus (Venezuela), Sabiá virus (Brazil), Chapare and Machupo viruses (Bolivia), and Junín virus (Argentina).

infection remains unknown. The patient was transferred to Johannesburg, South Africa, for medical management. Three secondary infections and one tertiary infection were reported, from which only one person survived.[3]

New World Mammarenaviruses

Argentinian hemorrhagic fever (AHF) cases were first described in the humid Pampas of Argentina in 1955 (Figures 21-1 and 21-2).[4] However, AHF epidemics may have occurred as early as 1943. The etiologic agent of AHF—JUNV—was later isolated from humans.[5,6] Since the 1950s, JUNV is estimated to have caused about 30,000 AHF cases. Without treatment, the case fatality rate is approximately 20%. The AHF-endemic region has expanded progressively into north-central Argentina to the extent that currently 5 million people are considered to be at risk of infection.[7]

Bolivian hemorrhagic fever (BHF) was recognized in 1959 on the island of Orobayaya in the Beni Department in northeastern Bolivia (Figures 21-1 and 21-3). However, it was not until 1964, after initial outbreaks of this emerging hemorrhagic fever (1959–1962) caused 470 cases, that BHF was first described by Mackenzie and coworkers.[8] MACV, the etiological agent of BHF, is named after a river close to the outbreak area. MACV was isolated in 1963 from the spleen of a fatal human case in San Joaquín.[9] Between 1962 and 1964, another series of localized BHF outbreaks occurred, which involved more than 1,000 patients, of whom 180 died. After 20 years of no reported cases, mainly as a result of rodent control measures,[9] an outbreak of 19 cases occurred in 1994. Eight additional BHF cases were recognized in 1999, and 18 cases occurred in 2000. A larger outbreak, with 200 suspected cases, occurred in 2008.[10] The case fatality rate of BHF is approximately 5% to 30%.

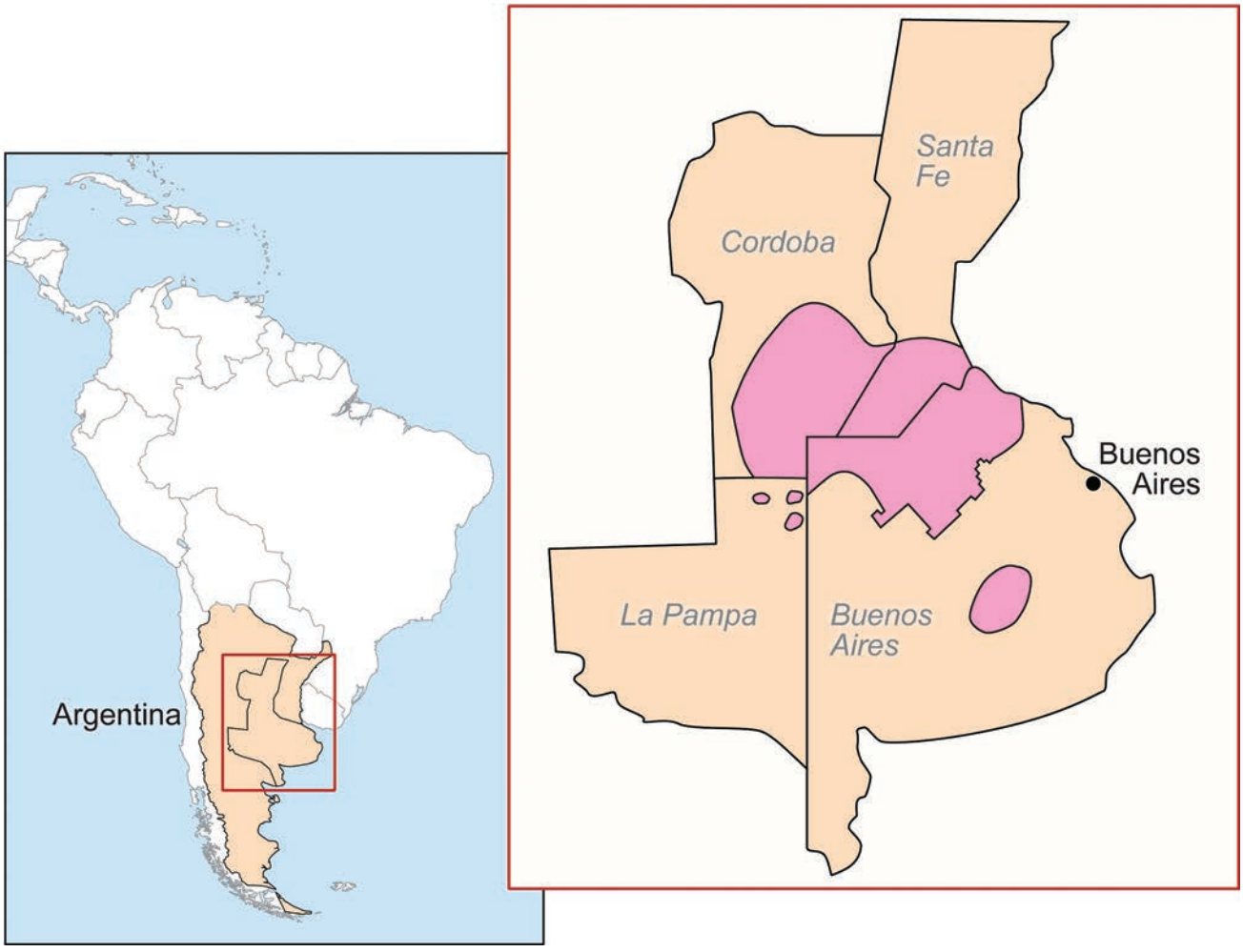


Figure 21-2. Geographic distribution of Junín virus in Argentina. Hyperendemic areas are shown in pink.

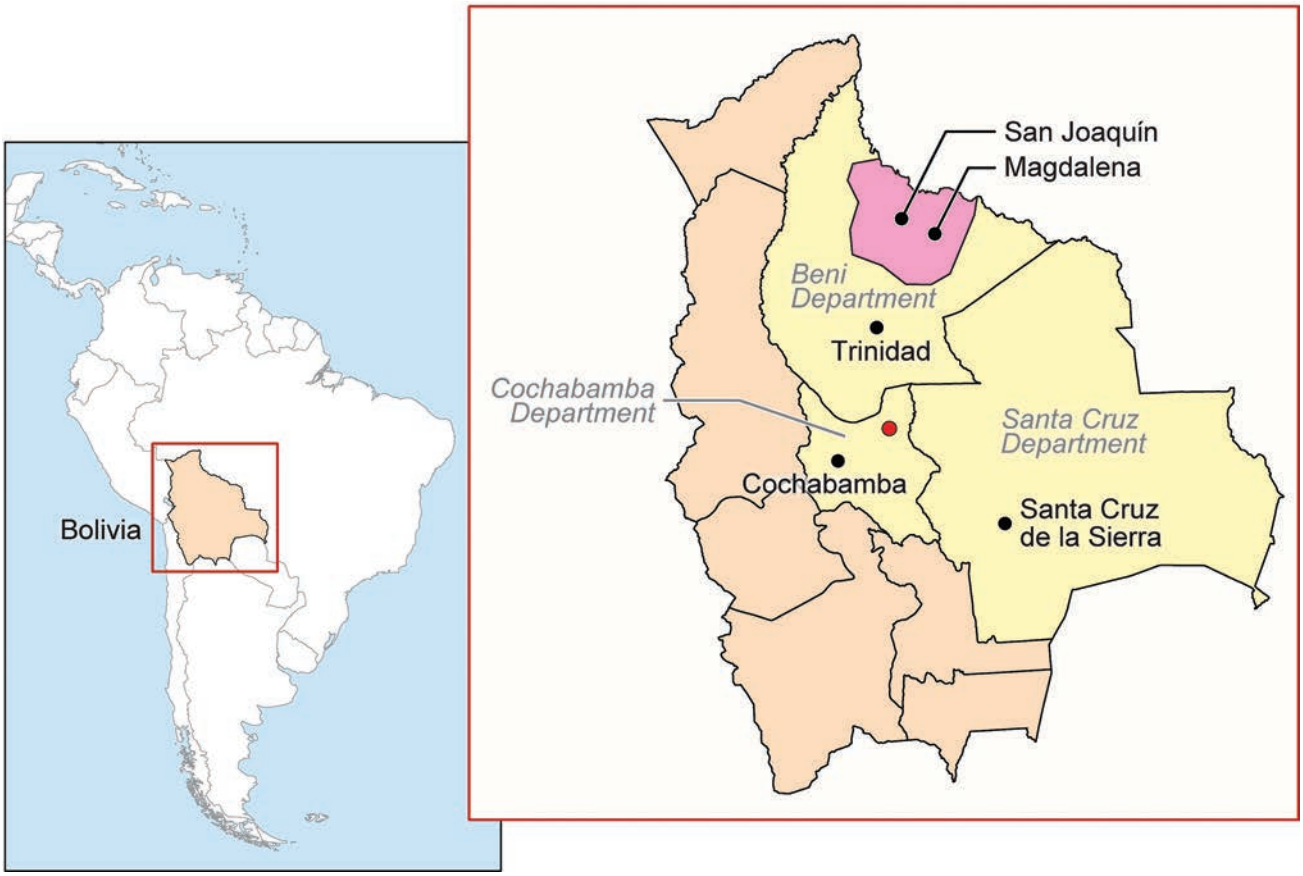


Figure 21-3. Geographic distribution of Machupo virus and Chapare virus in Bolivia (*yellow*). Hyperendemic areas are shown in pink. The only known location of Chapare virus infection is shown as a *red dot*.

GTOV emerged in 1989 as the cause of a yet officially unnamed disease that is often referred to as "Venezuelan hemorrhagic fever (VHF)." This severe hemorrhagic illness was recognized when settlers moved into cleared forest areas in the municipalities of Guanarito and Guanare in the state of Portuguesa in central Venezuela (Figures 21-1 and 21-4). The outbreak was initially misdiagnosed as severe dengue. Between 1990 and 1991, a total of 104 cases was reported with an approximately 25% case fatality rate.[11] The virus was isolated from the spleen of a 20-year-old male farm worker during autopsy.[12] After a seemingly spontaneous drop in human cases between 1989 and 1992, a new outbreak occurred in 2002 with 18 reported cases.[13] By 2006, approximately 600 cases of "VHF" have been reported.[14,15]

SABV, the cause of "Brazilian hemorrhagic fever," was isolated in 1990 from a single patient with a fatal infection in São Paulo, Brazil (Figure 21-1).[16] Subsequently, two laboratory infections were reported, one of which was successfully treated with ribavirin.[17]

In 2003 to 2004, CHAPV was recovered from a single fatal case of viral hemorrhagic fever in the Chapare River region in rural Bolivia (Figures 21-1 and 21-3). Additional cases were reported from this outbreak; however, details and laboratory confirmation are lacking.[18]

RESERVOIRS OF HEMORRHAGIC FEVER MAMMARENAVIRUSES

Rodents of the superfamily Muroidea are the natural hosts of most mammarenaviruses. Old World mammarenaviruses are found in rodents of the family Muridae, subfamily Murinae. New World mammarenaviruses are found in rodents of the family Cricetidae, subfamily Sigmodontinae. Bats may transmit Tacaribe virus, and reservoirs for Chapare, Lujo, and Sabiá viruses have not yet been identified.

The range of the corresponding rodent/bat host(s) determines the geographical distribution of each mammarenavirus. Field studies strongly support the concept of a single major reservoir host for each

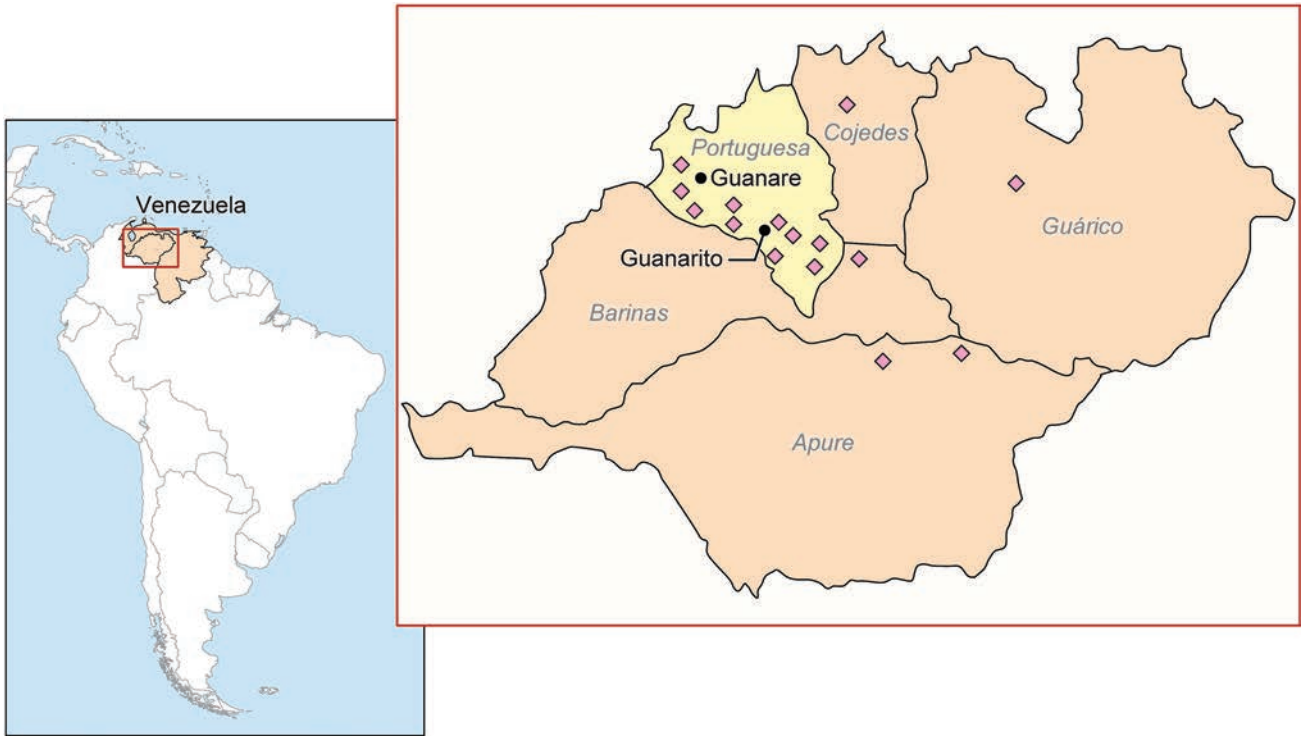


Figure 21-4. Geographic distribution of Guanarito virus in Venezuela. Clusters of infections areas are shown in *pink*.

virus.[19] Principal hosts for LASV, MACV, JUNV, and GTOV are the natal mastomys (*Mastomys natalensis*), the big laucha (*Calomys callosus*), the drylands laucha (*Calomys musculinus*), and the short-tailed zygodont (*Zygodontomys brevicauda*), respectively.[2,20–23]

Current evidence suggests a long-term "diffuse co-evolution" between mammarenaviruses and their rodent hosts. According to this model, a parallel phylogeny between the viruses and their corresponding rodent host(s) allows for host switches between closely related rodents.[19,24] Mammarenaviruses establish chronic infections in their respective reservoirs accompanied by chronic viremia or viruria without clinical signs of disease.[25–28]

Humans become infected with mammarenaviruses through contact with infected rodents or inhalation of aerosolized virus from contaminated rodent blood, excreta or secreta, or body parts caught in mechanical harvesters.[13] In Western Africa, peridomestic rodents are also part of the diet of inhabitants of LASV-endemic areas, and therefore contaminated meat may be another route of virus transmission.[29] New World mammarenavirus infections peak during harvest season when rodent populations are active. Infected cases are predominantly male agricultural workers who come in contact with infected rodents. Person-to-person transmission of LASV or MACV is not frequent, but it is possible by direct contact with body fluids or excreta of infected patients. Such transmission is probably not the principal mode of disease dissemination. Only small quantities of MACV can be isolated from human blood or from throat or oral swabs of infected patients.[30]

CLINICAL PRESENTATION

Old World Mammarenaviral Hemorrhagic Fevers

The signs and symptoms of Lassa fever vary depending on the disease's severity.[31,32] The disease is mild or asymptomatic in about 80% of infected people, but 20% develop acute Lassa fever. The incubation period can range from 1 to 24 days with an average of 7 to 18 days. Disease onset is insidious with low-grade fever, weakness, and general malaise. Within 2 to 4 days, many patients experience symptoms including myalgia; arthralgia; lower back, abdominal, and/or retrosternal pain; headache; dizziness; or sore throat. Hypotension, productive cough, vomiting, and diarrhea are also common. Pharyngitis or conjunctivitis can occur as the disease progresses, and mucosal bleeding (gums, nose, and other sites), pleural or

pericardial effusions, or facial or neck edema occur in more severe cases.[33] In the second week after onset, acute respiratory distress syndrome, moderate-to-severe diffuse encephalopathy, or shock develops in severe cases. Confusion, followed rapidly by tremors, convulsions, abnormal posturing, or coma, sometimes occur just before death.[34] Another neurological manifestation is unilateral or bilateral sensorineural deafness, which occurs in about 30% of convalescent patients.[33,35]

Lassa fever presents with symptoms and signs indistinguishable from those of other febrile illnesses, such as malaria or other viral hemorrhagic fevers. Therefore, Lassa fever is difficult to diagnose clinically, but it should be suspected in patients with fever (≥38°C) not responding adequately to antimalarial and antibiotic drugs. Fever, pharyngitis, retrosternal pain, and proteinuria are the most useful clinical predictors for a Lassa fever diagnosis. Fever, sore throat, and vomiting are the best predictors for negative outcome.[33] Disease outcome is also related to the degree of viremia and not to antibody response. The probability of fatal disease increases with high viremia, and survival rate is lowest in patients with both high viremia and high concentrations of aspartate aminotransferase. In patients recovering from Lassa fever, virus is cleared from blood circulation about 3 weeks after onset of illness.[36] Survivors of LASV infection often recover without sequelae. However, severe sensorineural hearing deficits, which may develop during disease, may persistent permanently in approximately 13% to 30% of survivors.[37–39]

Patients infected with LUJV initially present with symptoms and signs of nonspecific febrile illness such as severe headache, malaise, vomiting, fever, retrosternal pain, or myalgia. Disease manifestations increase in severity over 7 days with the development of diarrhea or pharyngitis. In some patients, morbilliform rash or facial edema is evident. Terminal features are acute respiratory distress syndrome, cerebral edema, neurologic signs, deteriorating renal function, or circulatory collapse. No overt hemorrhage is observed besides gingival bleeding, petechial rash, or oozing from injection sites in some of the patients.[3] However, the clinical description of disease caused by LUJV infection is currently based on the observation of only five patients.

New World Mammarenaviral Hemorrhagic Fevers

New World mammarenaviral hemorrhagic fevers caused by CHAPV, GTOV, JUNV, MACV, or SABV are clinically similar.[4,15,18,40–43] Disease begins insidiously after an incubation period of 1 to 2 weeks. Initial symptoms/signs often include fever and malaise, headache, myalgia, epigastric pain, or anorexia. After 3 to 4 days, signs become increasingly severe with multisystem involvement: prostration; abdominal pain; nausea and vomiting; constipation; or mild diarrhea. In some cases, dizziness, photophobia, retroorbital pain, or disorientation may also appear, as well as the earliest signs of vascular damage, such as conjunctival injection, skin petechiae, mild (postural) hypotension, or flushing over the head and upper torso. About 30% of patients develop more severe hemorrhagic or prominent neurologic manifestations (convulsions, tremor of the hands or tongue, coma) or secondary bacterial infections during the second week of illness. Hemorrhagic manifestations, such as bleeding from mucous membranes (gums, nose, vagina/uterus, gastrointestinal tract) and ecchymoses at needle puncture sites, are common in these patients. However, blood loss is minor overall. Capillary leakage is a hallmark of disease, and elevated hematocrit occurs during the peak of capillary leak syndrome.[13] Death usually occurs 7 to 12 days after disease onset from organ failure and shock.

Patients who survive begin to improve during the second week of disease onset. Convalescence often lasts several weeks with fatigue, hair loss, dizziness, or Beau's lines in digital nails. "VHF" convalescent symptoms also include sore throat or pharyngitis.[15,44,45]

TAXONOMY AND PHYLOGENETIC RELATIONSHIPS

The family *Arenaviridae* includes two genera, *Mammarenavirus* (mammalian arenaviruses) and *Reptarenavirus* (reptilian arenaviruses). The genus *Mammarenavirus* includes 31 species, and the majority of these species have only a single virus member each.[46,47] Based on antigenic properties (serological cross-reactivity), sequence phylogeny, and geographical distribution, mammarenaviruses have been divided into two distinct groups. The Old World mammarenaviruses (also named the Lassa–lymphocytic choriomeningitis serocomplex) include viruses indigenous to Africa, such as LASV and LUJV, as well as the ubiquitous lymphocytic choriomeningitis virus. The New World mammarenaviruses (also named the Tacaribe serocomplex) include viruses indigenous to the Americas,[48–51] such as CHAPV, GTOV, JUNV, MACV, and SABV.

The basis for mammarenaviruses phylogenetic analysis typically relies on the sequence of the nucleoprotein (N) gene. This analysis supports the previously defined antigenic grouping, further defines

virus relationships, and is largely consistent with analyses based on sequence data derived from other regions of mammarenaviruses genomes. According to N-based phylogenetic analysis, the member viruses of the 31 species represent four distinct phylogenetic groups: an Old World mammarenaviruses group and three New World mammarenavirus lineages (A, B, and C).[52–55] New World mammarenaviruses Group A includes Allpahuayo virus, Flexal virus, Paraná virus, Pichindé virus, and Pirital virus from South America, together with Bear Canyon virus, Tamiami virus, and Whitewater Arroyo virus from North America. Group B contains the human pathogenic viruses CHAPV, GTOV, JUNV, MACV, and SABV, and the nonpathogenic Amaparí virus, Cupixi virus, and Tacaribe virus. Group C includes Latino virus and Oliveros virus.

MOLECULAR CHARACTERISTICS

Arenaviruses produce enveloped and spherical to pleomorphic virions, ranging from 50 to 300 nm in diameter (Figure 21-5).[13,56–58] The particles' sandy appearance in electron microscopy sections earned these viruses their name (Latin arena = sand). The arenavirus genome consists of two single-stranded ribonucleic acid (RNA) molecules, designated L (large) and S (small). Each of these genomic segments encodes two different proteins in two nonoverlapping reading frames of opposite orientation (ambisense coding arrangement; Figure 21-5). The L segment encodes the viral RNA-dependent RNA polymerase (L) and a zinc-binding matrix protein (Z). The S segment encodes a nucleoprotein (NP) and an envelope glycoprotein precursor (GPC).[59–61] Extracted virion RNA is not infectious, and, therefore, arenaviruses are considered by some as negative-sense RNA viruses despite the presence of the ambisense coding strategy.

Arenavirus cell entry and fusion with the host membrane is mediated by the arenavirion spike complex (Figure 21-5). In the case of mammarenaviruses, the spike is composed of the two envelope glycoprotein subunits, GP1 and GP2, derived from posttranslational cleavage of GPC and a stable signal peptide, cleaved off during GPC synthesis.[61–65] Reptarenavirus spikes are fundamentally different from mammarenaviral spike proteins and are closely related structurally to the glycoproteins of filoviruses ($GP_{1,2}$). Therefore, a stable signal peptide is absent.[66] To enter cells, arenaviral GP1 binds to cell-surface receptors, and virions are internalized by endocytosis into intracellular endosomal compartments.[67–71] Following pH-dependent membrane fusion mediated by GP2 and uncoating, viral ribonucleoprotein (RNP) complexes are released into the cytoplasm (Figure 21-5).[58]

Interestingly, genes required for the proper functioning of α-dystroglycan (αDG), the receptor for Lassa virus (LASV), are preferential targets of LASV-driven selective pressure (or natural selection) in populations of Western Africa where LASV is endemic. A genome-wide screen for recent selective sweeps in humans has identified positive selection of two genes

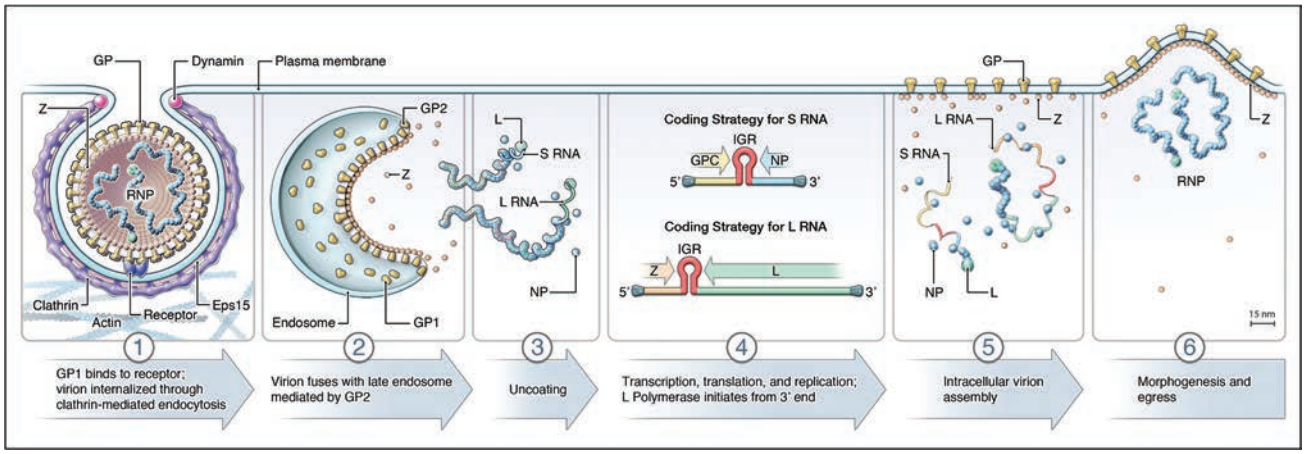


Figure 21-5. Replication cycle of an idealized mammarenavirus.
GPC: glycoprotein precursor
IGR: intergenic region
NP: nucleoprotein
RNA: ribonucleic acid

(LARGE and dystrophin) in a Nigerian population.[72–74] Dystrophin is a cytosolic adaptor protein that is necessary for αDG to function properly. The cellular like-acetylglucosaminyltransferase (LARGE) is required for the posttranslational modification of αDG and consequently for LASV GP binding to αDG.[75] In the identified population, more than 21% of individuals showed evidence of exposure to LASV infection, which may be responsible for the increase in the allelic variants of LARGE and dystrophin. Positive selection in LARGE was confined to introns and putative regulatory regions, suggesting that natural selection may have targeted variants giving rise to alternative splicing or differential gene expression of LARGE. These polymorphisms in LARGE and dystrophin may alter the ability of LASV GP to bind αDG and mediate entry, providing an advantage to the immune system, and thereby protecting these individuals from severe LASV infection.[72–74]

RNPs consist of NP, L, and viral genomic RNA. NP is the arenavirus major structural protein. It forms a bead-like polymer that associates with viral RNA. L, the arenaviral RNA-dependent RNA polymerase, mediates RNA replication and transcription (Figure 21-5).[76–79] L initiates transcription from the genome promoter at the genome 3′ end, and NP and L genomic complementary mRNA are synthesized and translated to proteins from the S and L segments, respectively.[80–82] L together with NP also generate a full-length copy of antigenome RNA from the L and S segments. Antigenome S and L RNA segments then serve as a template for the synthesis of GP and Z mRNAs, which are translated into the respective proteins. Newly synthesized full-length arenavirus antigenomic and genomic RNAs are encapsidated by NP to generate the RNP complexes for further mRNA transcription and for production of virus progeny (Figure 21-5).[83] The negative regulatory matrix protein Z inhibits arenaviral RNA synthesis in a dose-dependent manner.[82,84–88] Z contains a zinc-binding RING motif[89,90] that is essential for interaction with L and resultant inhibitory activity.[86]

Together with NP, Z mediates arenavirion assembly[91] and budding.[80,91,92] Virion budding occurs from the plasma membrane, where the virus RNP core associates with host-derived membrane that is highly enriched with the viral GP spike complex to form the virion envelope (Figure 21-5).[80,91–93]

In addition to the roles of NP and Z in viral replication, these proteins interfere with antiviral signaling. NP encoded by Old World and New World mammarenaviruses is involved in virus-induced inhibition of type I interferon.[94,95] Z protein encoded by New World mammarenaviruses also interferes with this pathway.[96]

PATHOLOGY AND PATHOGENESIS

The mechanism of pathogenesis of mammarenaviral hemorrhagic fevers is not well understood. In both Old World and New World mammarenavirus infections, the pathological findings do not provide the basis to explain the relatively high case fatality rate and severity of disease.[11,97–101] According to the current pathogenesis model, mammarenaviruses enter humans by inhalation and deposit in the lung terminal respiratory bronchioles. The viruses then gain entry to the lymphoid system and spread systematically in the absence of a detectable pneumonic focus.[102] Mesothelial surfaces are infected next, perhaps a source of some of the observed effusions of parenchymal cells of several organs, particularly lymphoid tissues. Macrophages are early and prominent targets of mammarenavirus infection.[17,103,104]

Old World Mammarenaviral Hemorrhagic Fevers

In the case of patients infected with LASV, failure to develop cellular and humoral immune responses, indicated by high levels of serum virus titers and virus replication in tissues, leads to the development of fatal Lassa fever.[105] Nonhuman primate models of Lassa fever indicate that dendritic cells are prominent targets of LASV in the initial stages of infection, whereas Kupffer cells, hepatocytes, adrenal cortical cells, and endothelial cells are more frequently infected with LASV in the terminal stages of infection.[106]

Macroscopic abnormalities in Lassa fever patients include pleural effusions, pulmonary edema, ascites, and hemorrhagic manifestations in the gastrointestinal mucosa.[1,31] Microscopic findings include multifocal hepatocellular necrosis and apoptosis or regeneration (mitosis), splenic necrosis in the marginal zone of the splenic periarteriolar lymphocytic sheath, adrenocortical necrosis, mild mononuclear interstitial myocarditis without myocardial fiber necrosis, alveolar edema with capillary congestion and mild interstitial pneumonitis, lymph nodal sinus histiocytosis, gastrointestinal mucosal petechiae, renal tubular injury, or interstitial nephritis.[1,31,107,108]

The most severe pathological hallmark of Lassa fever in humans is found in the liver.[1,31,107,109] In addition to hepatocellular necrosis, mononuclear phagocytosis of necrotic hepatocytes and focal hepatocellular cytoplasmic degeneration are typical. However, the degree of hepatic tissue damage is insufficient to cause hepatic

failure, and only minimal recruitment of inflammatory cells into this organ is detected. Furthermore, no correlation has been observed between the degree of hepatic necrosis and chemical indicators of liver damage, such as elevated concentrations of aspartate aminotransferase, alanine transaminase, and lactate dehydrogenase in serum.[107,108] Finally, the degree of liver damage can vary dramatically among patients that die from Lassa fever. Therefore, liver involvement is necessary—but not sufficient—in the chain of pathological events that lead to fatal Lassa fever.

Based on the degree of hepatic damage, three general phases have been proposed for the categorization of patients with fatal Lassa fever.[66] The first phase, active hepatocellular injury, is defined by the presence of focal cytoplasmic degeneration with less than 20% of hepatocytes undergoing necrosis. This phase may represent the late stage of viremic spread and early cellular injury. This phase is most likely caused by direct viral action rather than mediated by a cellular immune response, since lymphocytic infiltration is not detected. The second phase, the peak of Lassa hepatitis, is characterized by 20% to 50% necrosis of hepatocytes, widespread focal cytoplasmic degeneration, and limited phagocytic infiltration. Progressive hepatocellular damage occurs during this phase, but early liver recovery is evident through the phagocytic removal of necrotic hepatocytes and regeneration of new cells. The third phase, hepatic recovery, is defined by less than 10% of hepatocellular necrosis, absence of focal cytoplasmic degeneration, and clear evidence of mitoses, which indicates liver regeneration.[108]

In addition to hepatic necrosis, splenic and adrenocortical cellular necrosis is observed in patients with Lassa fever. The predominant distribution of splenic necrosis is in the marginal zone of the periarteriolar lymphocytic sheath. Close examination of thin tissue sections reveals the presence of fibrin in addition to the debris of necrotic cells. The splenic venous subendothelium appears to be infiltrated by lymphocytes and other mononuclear cells. Additionally, multifocal adrenocortical cellular necrosis is most prominent in the zona fasciculata and is often associated with focal inflammatory reaction. However, in all examined cases, adrenal necrosis was mild, and greater than or equal to 90% of the cells of adrenal cortex appeared viable.[107] Microscopic examination of adrenal glands shows prominent spherical, hyaline, and acidophilic cytoplasmic inclusions in cells near the junction of zona reticularis and medulla. In most cases, these cells appear to be adrenocortical cells of the zona reticularis; however, some cells are of adrenal medulla origin.

LASV-induced impairment of vascular function is most likely central for the pathology observed in infected patients. LASV, which is a nonlytic virus, does not cause cytopathic effects or cellular damage in infected monocytes, macrophages, and endothelial cells.[110] Nevertheless, infection of these cells is crucial for the pathology caused by the virus. In both experimentally infected animals and Lassa fever patients, the disruption of vascular endothelium function is closely followed by shock and death.[107,111] Edema and pleural and pericardial effusions that are associated with fatal cases most likely result from increased vascular permeability. Only minimal vascular lesions are detected in fatal human Lassa fever cases and infected nonhuman primates, which can be explained by the nonlytic nature of LASV infection of the endothelium. The mechanism of LASV-induced increase in vascular permeability is not yet understood. Virus infection of the endothelium is commonly thought to cause changes in cellular function leading to increased fluid flow and subsequently to edema.

New World Mammarenaviral Hemorrhagic Fevers

The most common macroscopic abnormality in severe cases of New World mammarenaviral hemorrhagic fevers is widespread hemorrhage, particularly in the skin and mucous membranes (gastrointestinal tract), intracranium (Virchow-Robin space), kidneys, pericardium, spleen, adrenal glands, and lungs. Microscopic lesions include acidophilic bodies and focal necroses in the liver (in the case of BHF, hepatic petechiae are common, and the number and size of the Kupffer cell are also increased), acute tubular and papillary necrosis in the kidneys, reticular hyperplasia of the spleen and lymph nodes, or secondary bacterial lung infections in the case of AHF (acute bronchitis and bronchopneumonia, myocardial and lung abscesses) or interstitial pneumonia in the case of BHF[97,98] or "VHF."[11,112] In AHF, the sites of cellular necrosis (hepatocytes, renal tubular epithelium, macrophages, dendritic reticular cells of the spleen and lymph nodes) correspond to sites of viral antigen accumulation, and both JUNV and MACV could be isolated from the blood, spleen, and lymph nodes of patients.[20,30,113,114]

Patterns of clinical AHF illness are JUNV strain-specific and can be hemorrhagic (Espindola strain), neurologic (Ledesma strain), mixed (P-3551 strain), and common (Romero strain).[115–117] In animal models of AHF, each isolate induces a disease that faithfully replicates the clinical variant of the disease in the human from whom the viral strain was obtained. Animals infected with JUNV Espindola strain (hemorrhagic) demonstrate a pronounced bleeding tendency with

disseminated cutaneous and mucous membrane hemorrhage. In contrast, animals infected with JUNV Ledesma strain (neurologic) show little or no hemorrhagic manifestations, but develop overt and generally progressive signs of neurologic dysfunction: limb paresis, ataxia, tremulousness, or hyperactive startle reflexes. In guinea pigs, the Espindola strain replicates predominantly in the spleen, lymph nodes, and bone marrow, the major sites of necrosis, whereas lower virus loads are present in blood and brain. The Ledesma strain, however, is found predominantly in the brain, where moderate polioencephalitis is observed, and only low amounts of virus are recovered from the spleen and lymph nodes.

COAGULOPATHIES

Although the mammarenaviruses discussed in this chapter cause viral hemorrhagic fevers in humans, blood loss does not typically account for the diseases' fatal outcome.[107] Furthermore, pathogenic mammarenaviruses differ in their capacity to cause hemorrhages or coagulopathies in infected individuals, which is particularly true in Lassa fever patients in whom bleeding is uncommon and limited primarily to mucosal surfaces.[118] In general, coagulation dysfunction is not considered to be associated with Lassa fever as neither disseminated intravascular coagulation nor a decrease in coagulation factors has been observed.[119] However, in the few severe cases characterized by bleeding, it is typically associated with moderate thrombocytopenia and platelet dysfunction.[119,120] The platelet malfunction appears to be mediated by a plasma inhibitor of platelet aggregation, which has yet to be characterized.[120]

Hemorrhages are more common in patients infected with New World mammarenaviruses than with Old World mammarenaviruses. However, similar to Lassa fever patients, relatively few vascular lesions are seen in patients with AHF.[121] Endothelial cells, which highly express the New World mammarenavirus receptor transferrin receptor 1, are permissive to infection in culture, but minimal cytopathic effects are observed.[70,122,123] Therefore, indirect effects may be responsible for the increased permeability seen in patients,[104] and profuse bleeding is presumably a consequence of vascular damage caused by both cytokines and virus replication. Thrombocytopenia, which is commonly found in patients with AHF and in animals experimentally infected with JUNV or MACV, and elevated concentrations of factor VIII-related antigen (von Willebrand factor, vWF), which is synthesized and released from endothelial cells, could contribute to the observed endothelial dysfunction.[124–128] However, vWF concentrations are low in JUNV-infected human umbilical vein endothelial cells, suggesting that vWF might originate from another source rather than from endothelial cells. Human umbilical vein endothelial cells infected with virulent JUNV strains increase production of the vasoactive mediators nitric oxide and prostaglandin PGI_2 compared to that observed with avirulent strains, providing a possible link between viral infection and the increased permeability observed in patients with AHF.[123]

In animal models of AHF and BHF, progressive thrombocytopenia is observed within 7 days following infection onset, with platelet counts reaching a nadir at or near the time of death. Coincident with the dropping platelet count, progressive necrosis of bone marrow occurs, suggesting that the decrease in the number of platelets may be related to impaired production.[101,117,127,128] Furthermore, intracytoplasmic viral particles have been demonstrated in megakaryocytes by electron microscopy.[129] The coagulative activity of blood in infected patients with AHF is also low.[130] Similar to Lassa fever patients, plasma from patients with AHF contains an inhibitor of platelet function.[35] Platelet inhibition appears to be reversible in vitro,[131,132] and its effects are not neutralized by immune plasma containing a high titer of (neutralizing) antibodies to JUNV. Thus, the available evidence suggests that abnormal platelet function in patients does not result from an intrinsic platelet defect, but rather from inhibition by an extrinsic factor in plasma.

Overall, specific coagulation abnormalities do not correlate with the severity of New World mammarenaviral infections, suggesting minimal involvement of coagulopathy in pathogenesis. Furthermore, limited evidence (four human cases)[98,133] suggests that disseminated intravascular coagulation is not an important pathogenic phenomenon in mammarenaviral disease. However, several modest abnormalities of clotting factors and activation of fibrinolysis are observed in AHF patients and animal models. These abnormalities can vary depending on which virus variant is involved.[124–126,128,134–136] The concentration of factor V is uniformly elevated (starting from day 8 of onset of AHF), and fibrinogen concentration is normal in mild cases and elevated in severe cases in the later stages of infection (after day 10). The activated partial thromboplastin time is prolonged during the acute phase of illness. A lower concentration ratio of factor VIII:C to vWF has been noted during the illness, but returns to normal during the convalescence period. In the guinea pig model, and to a lesser extent in humans, factor IX and XI concentrations are slightly

reduced.[124,126–128,130,134] Levels of prothrombin fragment 1+2 and thrombin-antithrombin III complexes are increased. However, antithrombin III activity levels in patients with AHF are within the normal range.[130] In another study in patients with AHF, antigenic and functional levels of antithrombin III were below normal in the early stages of disease.[137] In most cases of AHF, no significant changes in factor II, VII, prekallikrein, and kallikrein inhibitor, protein C, protein S, and C4b binding protein are observed.[124,130] Plasminogen activity is below normal in AHF patients in the earlier stages of the disease (days 6–11),[138] although normal or slightly elevated concentrations of α_2-antiplasmin can be detected.[124,130] Tissue plasminogen activator and D-dimer concentrations are high in the early stages of the disease, whereas the plasminogen activator inhibitor-I concentration is increased only in severe cases during the second week of illness.[130]

IMMUNE RESPONSE

Old World Mammarenaviral Hemorrhagic Fevers

Antibodies do not seem to play a significant role in LASV infection because production of LASV-specific antibodies is not correlated with Lassa fever survival. Such antibodies are detected in all patients, regardless of outcome.[36] Low neutralizing antibodies titers occur only months after an acute LASV infection is resolved, long after virus has been cleared.[139] Instead, resolution of LASV infection seems to depend primarily on cellular immunity, in particular the antiviral T-cell response.[140,141] Data from experimental nonhuman primate studies show a correlation between surviving animals and high concentrations of activated T lymphocytes and control of viral replication. In contrast, animals that died had delayed, low T-cell activation and uncontrolled viral replication.[142] In addition, seropositive individuals residing in Lassa fever-endemic areas have very strong memory $CD4^+$ T-cell responses, and the antigenic epitopes have been mapped to NP and an N-terminal conserved region within GP2.[141,143] However, results of a recent study using mice expressing humanized major histocompatibility complex class I and a single, exotic variant of LASV (Ba366) suggest that in the presence of persistent viremia, T cell responses may also contribute to deleterious innate inflammatory reactions and Lassa fever pathogenesis.[105] Whether these results can be generalized to other LASV variants remains to be determined.

In contrast to other viral hemorrhagic fevers, such as Ebola virus disease,[144] LASV infection does not result in a "cytokine storm" that interferes with the integrity of the vascular endothelium.[145] Virus-induced immunosuppression may be involved in the pathogenesis of severe Lassa fever. Antigen-presenting cells, such as dendritic cells and macrophages, and endothelial cells are early targets of LASV infection, with dendritic cells producing more virus than macrophages. However, these cell types are not activated to produce proinflammatory cytokines, including tumor necrosis factor-α (TNF-α) and interleukin-8 (IL-8).[110,146,147] These results are consistent with clinical data from Lassa fever patients showing correlation of fatal outcome and low concentrations or absence of proinflammatory cytokines such as IL-8 and interferon (IFN)-inducible protein 10.[145] Although increased vascular permeability does not seem to be caused by inflammatory mediators, LASV infection may affect endothelial cell integrity via another mechanism.

New World Mammarenaviral Hemorrhagic Fevers

Similar to Lassa fever, the acute phase of New World mammarenaviral hemorrhagic fevers is associated with significant depression of host immunity. The frequency of pyogenic secondary bacterial infections in humans and animal models[97,98,115] suggests that polymorphonuclear leukocyte function is compromised. Leukocyte dysfunction may be a result of bone marrow necrosis, maturation arrest, and direct interactions of JUNV with polymorphonuclear cells[148] that lead to leukopenia.[149,150] AHF is associated with a profound decrease in the recall of delayed hypersensitivity, diminished responsiveness of lymphocytes to nonspecific mitogens, decreased levels of circulating B- and T-cells, lymphoid necrosis, and inversion of $CD4^+/CD8^+$ lymphocyte ratios.[114,151–154] Abnormalities reported in animal models include necrosis of macrophages, T- and B-lymphocyte depletion, decreased primary and secondary antibody responses, and blunted Arthus reaction and anergy after established tuberculin sensitivity.[20,129,149,155–157] Defective macrophage function and high concentrations of IFN are highly plausible causes for these observed abnormalities. JUNV infects macrophages and monocytes extensively in vivo,[114] and circulating monocytes contribute to viral spread in the acute phase of AHF.[158] Virulent JUNV strains replicate in both spleen-derived dendritic cells and macrophages from guinea pigs, whereas attenuated strains, which are not immunosuppressive, replicate only in dendritic cells.[159]

Unlike LASV infection, in which generalized immune suppression is observed, AHF patients have elevated concentrations of proinflammatory as well as antiinflammatory cytokines that correlate with the disease's severity. Both patients and animal models

have high serum concentrations of IFN-α. The extraordinarily high serum concentrations of IFN-α in JUNV-infected patients are indicative of a negative disease outcome.[160,161] In patients who survive, high concentrations of IFN-α only occur during the first week after disease onset and fall to low concentrations during the second and following weeks. However, concentrations of interleukin-1β (IL-1β) remain normal.[102,160–162]

High serum concentrations of TNF-α, IL-6, IL-8, IL-10, and elastase-α1-antitrypsin complex are found in patients with AHF. Significant correlations are found between concentrations of both IL-8 and IL-10 with TNF-α concentrations, and between IL-8 and elastase-α1-antitrypsin complex. Thus, IL-8 is suggested to play an essential role in neutrophil activation in AHF patients.[163] Elevated TNF-α concentrations may be the trigger for some of the observed hemostatic and endothelial abnormalities observed in AHF patients. Results of several studies characterize the procoagulant activity[164–166] and changes in vascular permeability[167–169] of endothelial cells following exposure to TNF-α. JUNV–infected macrophages show no increase in cytokine production indicating that perhaps dendritic cells may increase cytokine concentrations.[170] At present, it is unclear if the increased cytokine concentrations reflect high viral replication and widespread infection or if they play a role in immune-mediated pathology similar to other viral hemorrhagic fevers like Ebola virus disease.

In contrast to Lassa fever, in which the antibody response seems to be ineffective in controlling LASV infection, patients who recover from AHF develop antibodies in the second week of disease and clear the virus.[171] In convalescent plasma from AHF survivors, robust titers of neutralizing antibodies, mainly immunoglobulin G (IgG), can be detected. After transfusion with immune plasma, viremia in patients is reduced.[172] The case fatality rate of AHF patients treated early during the course of infection with immune plasma from surviving cases is reduced to 1%.[173]

DIAGNOSIS

Detection of Virus-Specific Antibodies and Viral Antigens

Mammarenavirus antibodies can be detected by enzyme-linked immunosorbent assays (ELISAs), virus neutralization tests, and indirect immunofluorescence assays (IFAs). ELISAs using recombinant proteins, infected cells, or blood as antigen have been developed for detection of pathogenic mammarenavirus antibodies.[174–177] An immunoglobulin M (IgM) or IgG-specific ELISA is suitable for determining exposure to mammarenaviruses, but the relevance of IgM or IgG testing for acute infection depends on the virus and duration of illness.[176] An early immunosuppression resulting from Lassa fever seems to result in depressed production of IgM at the early phase of the infection and, as a result, some patients fail to elaborate IgM at the time of presentation. However, an increase in IgG titers is observed during convalescence. Thus, neither IgM nor IgG titers alone should be used as a screening tool for early detection of Lassa fever.

The virus neutralization assay is accepted as a standard serodiagnostic assay to quantify the antibody response to infection of a wide variety of viruses.[176] However, this test can be used for diagnosis of mammarenavirus infections only if a biosafety level 3 or 4 laboratory is available. Virus neutralization tests are highly specific, but neutralizing antibodies may appear too late in the course of mammarenaviral disease to be useful for prompt diagnosis. For example, patients with Lassa fever do not usually develop neutralizing antibodies until weeks after they became ill,[178] and patients with fatal Lassa fever may not develop, antibodies at all.[179]

IFA tests also detect antibodies in serum that bind to a fixed monolayer of virus-infected cells.[33,180] However, the interpretation of IFA is complicated by positive staining results in both the acute- and convalescent-phase of infection, as well as the subjective nature of the assay.[174] ELISAs are thought to be more sensitive and specific than IFA.[174,175,181,182] Cross-reactions can occur between different arenaviruses in these tests.

Compared to antibody detection, antigen-capture ELISA using polyclonal or monoclonal antibodies for detection of viral antigens is valuable for rapid diagnosis of acute phase viral hemorrhagic fevers, such as Lassa fever, AHF, BHF, "VHF," and "Brazilian hemorrhagic fever."[174,183] The sensitivity of sandwich antigen-capture ELISA is comparable to that of reverse transcription polymerase chain reaction (RT-PCR) for detection of Lassa fever.[184] In a comparison of the diagnostic markers in a large cohort of potential Lassa fever patients, LASV antigens detected in blood using antigen-capture ELISAs or lateral flow immunoassays are more indicative of an acute LASV infection than positive antibody titers.[177]

Detection of Viral Nucleic Acids

RT-PCR, real-time PCR, and real-time RT-PCR tests are valuable tools for rapid and early diagnosis of mammarenavirus infections.[177,179,185–192] However, the use of these assays in a clinical or environmental setting for the early detection of human cases has been limited by the expense of equipment and by expertise. RT-PCR has been used routinely for

confirmation of Lassa fever in Africa during collaborative missions following antigen detection by ELISA and lateral flow immunoassay.[177,193] Some PCR tests detect a wide range of mammarenaviruses by targeting the highly conserved termini of the S RNA segment, but an RT-PCR assay detecting Old World mammarenaviruses targeting the L gene has been also developed.[194] Other PCR tests are more virus-specific. Serum, plasma, cerebrospinal fluid, throat washings, and urine can be used for sample preparation. Real-time PCR may be advantageous because the risk of contamination is greatly reduced by using closed tubes and because the test quantifies viral RNA in serum. However, specimens containing a high concentration of viral RNA may produce false-negative results resulting from inhibition of the enzymatic reaction.[178] Given the high degree of genetic variability of mammarenaviruses, selection of primers that can detect all strains of the viruses can be difficult,[176] and PCR techniques may fail to amplify sequences of mammarenavirus strains even with limited sequence deviations.

Virus Isolation

Virus isolation is the gold standard for diagnosis of mammarenavirus infections.[174,177,193] Mammarenaviruses can easily be recovered in cell cultures, particularly from Vero cells. Initial passaging of a virus isolate in laboratory rodents, such as suckling laboratory mice, guinea pigs, or newborn hamsters, may be even more sensitive. The presence of virus can then be confirmed by PCR or by detection of virus antigen in cells using immunohistochemical or IFA assays. However, considering the time required for virus isolation (days to weeks) and the need for special facilities (biosafety level 3 or 4 laboratories), which are unavailable in many mammarenavirus-endemic areas, this method is less suitable for rapid diagnosis of mammarenaviral disease than PCR or antigen-capture ELISA.[174,177,195] Recent discoveries of novel mammarenaviruses relied on the use of IFA, PCR, and pyrosequencing technology.[3,18,194,196,197] Next-generation sequencing technology may be used in the near future for diagnostic purposes.

TREATMENT AND VACCINES

Few prophylactic and therapeutic treatments are approved for use against mammarenaviral hemorrhagic fevers. Treatment, therefore, consists primarily of supportive care and passive antibody therapy.

Passive Antibody Therapy

Transfusion of immune convalescent plasma with defined doses of JUNV-neutralizing antibodies is the present therapeutic intervention and treatment method against AHF. Immune serum treatment providing an adequate dose of neutralizing antibodies is effective in attenuating disease and reducing lethality to less than 1% if administered within the first 8 days of disease.[173,198–200] However, about 10% of treated patients develop a transient cerebellar-cranial nerve syndrome 3 to 6 weeks later.[173,198–200]

Studies with animal models suggest that passive antibody therapy may be useful for the treatment of BHF,[201] but such therapy has not been thoroughly evaluated in a clinical setting. An in vitro study with Vero E6 cells shows that convalescent sera from 6 of 7 putative "VHF" cases neutralized the infectivity of GTOV, and the neutralizing titers in the positive sera range from 160 to 640.[14] However, even if a similar plasma therapy could be developed for BHF and "VHF," maintaining adequate plasma stocks would be a challenge because of the limited number of cases and the absence of a program for convalescent serum collection. The additional risk of transfusion-borne diseases emphasizes that alternative treatments ought to be developed.[202]

In contrast, treatment of Lassa fever patients with convalescent serum of survivors did not confer protection when treated within 24 hours after hospital admission.[203] Treatment of nonhuman primates and guinea pigs with plasma from convalescent animals containing high titers of neutralizing antibodies protects the animals from developing disease. However, protection is observed only if administration of plasma is performed directly after infection with LASV.[204,205]

Vaccines

Despite the bioterrorism and public health risks associated with pathogenic mammarenavirus infection, FDA-licensed vaccines are currently not available. Vaccines for the prevention of human mammarenavirus diseases are limited to a single, safe, efficacious, and live attenuated vaccine designated Candid 1 (Candidate no. 1), for the prevention of JUNV infection.[200,206–208] Candid 1, which is classified as an investigational new drug in the United States, was derived from the wild type JUNV strain XJ13 through serial passage both in vivo and in vitro.[209] A recent study suggests that the major determinant of attenuation in mice is located in the transmembrane domain of the G2 glycoprotein (F427I mutation).[210] Candid 1 has been evaluated in

large-scale controlled trials among at-risk populations of agricultural workers in Argentina, where it showed a protective efficacy greater or equal to 84%. Vaccination of more than 150,000 high-risk individuals in the endemic areas has led to a consistent reduction in AHF cases with an excellent safety profile.[208,211,212] The vaccine also cross-protects experimental animals against MACV infection,[213] which suggests that Candid 1 could be used during a BHF outbreak as an emergency containment measure. A summary of the historical development and biological properties of the vaccine can be found in a recent review.[214]

Another approach for vaccine development against AHF involves using a nonpathogenic mammarenavirus relative, Tacaribe virus, as a live vaccine in guinea pigs or the marmoset model of AHF.[215–218] Animals develop measurable immune responses as early as 3 weeks following exposure to Tacaribe virus, and no clinical signs of AHF or histopathological changes are observed following exposure to a lethal dose of JUNV.

Several promising studies have focused on the development and preclinical testing of LASV vaccines. Nevertheless, no vaccine candidate has advanced toward human clinical trials. Early strategies involved the usage of an apathogenic mammalian Old World mammarenavirus, Mopeia virus, as a live vaccine. Rhesus monkeys that were inoculated with Mopeia virus and subsequently exposed to LASV developed no sign of LASV disease.[219,220] However, since little is known about human infections with Mopeia virus, and some of the infected primates developed pathological alterations of the livers and kidneys,[221] the safety of Mopeia virus should be proved before any efficacy studies are performed in humans.

Another live attenuated vaccine candidate against LASV infection is the chimeric ML29 virus. This recombinant virus carries the LASV S segment and the Mopeia virus L segment and is efficacious in nonhuman primates. Immunity is conferred via cellular responses, and no transient elevation of liver enzymes in the plasma is noted.[222,223] However, as in the case with candidate vaccines based on Mopeia virus only, caution must be exerted, as the safety of LASV-Mopeia chimeric vaccines in humans is unclear.

Recombinant viruses expressing mammarenaviral antigens have also been tested as potential vaccines. Different viral platforms, such as vaccinia virus, vesicular stomatitis Indiana virus, attenuated yellow fever strain 17D virus, and Venezuelan equine encephalitis virus replicon particles expressing mammarenaviral NP, GP, GP1, or GP2, have been evaluated in various animal models.[140,224–230] The most promising results were obtained using the whole GP of LASV.

Other approaches based on inactivated mammarenaviruses[231,232] or mammarenavirus-like particles[233] have not been successful or have yet to be fully evaluated, respectively.

Antiviral Agents

Current antimammarenaviral therapy is limited to an off-label use of the nonimmunosuppressive guanosine analogue, ribavirin (1-β-D-ribofuranosyl-1-*H*-1,2,4-triazole-3-carboxamide), an IMP dehydrogenase inhibitor. Recent studies suggest that the antiviral activity of ribavirin on mammarenaviruses is not mediated by depletion of the intracellular GTP pool, but may be exerted—at least partially—by lethal mutagenesis.[234–236] Unfortunately, ribavirin has only partial efficacy against some mammarenavirus infections and is associated with significant toxicity in humans.[17,200,203,212,237–244] Ribavirin can lead to adverse side effects such as thrombocytosis, severe anemia, and birth defects.[241,245]

Promising antivirals have been identified by small-molecule high-throughput screens. These antivirals can be divided into six chemically distinct classes of small-molecule compounds that specifically inhibit GP-mediated membrane fusion with different selectivities against New World and/or Old World mammarenaviruses.[236,246–248] One highly active and specific small-molecule inhibitor, ST-294, inhibits MACV, JUNV, GTOV, and SABV at concentrations in the nanomolar range. This molecule also demonstrates favorable pharmacodynamic properties (metabolically stable, orally bioavailable) and in vivo anti-mammarenaviral activity in a newborn mouse model.[246] Mechanism-of-action studies suggest that this compound is a viral entry inhibitor targeting GP2.[246] Another compound, ST-193, a benzimidazole derivative, inhibits cell entry of MACV and GTOV in vitro.[236] Finally, two lead compounds, 16G8 and 17C8, are highly active against MACV and GTOV, as well as LASV. These compounds act at the level of GP-mediated membrane fusion (IC_{50} ≈200–350 nM).[247] Despite chemical differences, evidence suggests that these diverse inhibitors act through the pH-sensitive interface of the signal peptide and GP2 subunits in the GP spike complex. The inhibitors prevent virus entry by stabilizing the prefusion spike complex against pH-induced activation in the endosome.[236,246,248]

Other types of inhibitors that target viral RNA synthesis have also been reported. T-705 (favipiravir), a pyrazine derivative with broad antiviral activity against RNA viruses[249–251] and several nonpathogenic mammarenaviruses,[252,253] is also active in vitro against MACV, JUNV, and GTOV. T-705 most likely acts as

a purine nucleoside analog specifically targeting the viral RNA-dependent RNA polymerase.[254] Results of studies using the Pichindé virus hamster model of acute mammarenaviral disease or a guinea pig model with an adapted Pichindé virus demonstrate that T-705 could effectively protect against mammarenaviral disease after onset of clinical signs or in the late stage of illness.[252,255]

SUMMARY

Arenaviruses represent a large and taxonomically diverse group of animal viruses that are maintained by small rodents, bats, and snakes in nature. The majority of arenaviruses is not known to cause disease in humans. Seven mammarenaviruses, however, are the etiological agents of severe viral hemorrhagic fevers associated with high case fatality rates. LUJV, CHAPV, GTOV, and SABV, which are geographically restricted, have been associated with only a few to a few dozen cases. They are, therefore, relatively unimportant to clinicians or the warfighter compared to many other viruses that are usually coendemic. However, MACV, JUNV, and especially LASV have caused large outbreaks (LASV has caused hundreds of thousands of infections per year). Visitors to countries in which these viruses are endemic, or warfighters that are deployed to these countries, need to be aware of how to prevent and suspect a mammarenavirus infection. Distance from or safe handling of rodents and their bodily fluids or tissues and general rodent control around human settlements or camps should be the first priority to prevent mammarenavirus infections.

Acknowledgments

We are grateful for the technical writing services offered by Laura Bollinger (IRF-Frederick) and the support by IRF-Frederick's medical illustrator, Jiro Wada. The content of this publication does not necessarily reflect the views or policies of the US Department of Defense, US Department of Health and Human Services, the Department of the Army, or the institutions and companies affiliated with the authors. This work was funded in part through Battelle Memorial Institute's prime contract with the US National Institute of Allergy and Infectious Diseases under Contract No. HHSN272200700016I. A subcontractor to Battelle Memorial Institute who performed this work is JHK, an employee of Tunnell Government Services, Inc.

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Chapter 22

HENIPAVIRUSES

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SUMMARY

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INTRODUCTION

Henipaviruses were not discovered until 1994, when a novel virus, later called Hendra virus (HeV), was identified as the etiological agent of a fatal viral encephalitis in Hendra, Australia. Nipah virus (NiV), another virus closely related to HeV, emerged in Malaysia in 1998. Subsequently, the genus *Henipavirus* was established within the family *Paramyxoviridae* as a taxonomic home for both HeV and NiV. HeV and NiV are high-consequence pathogens that are thought to be potential starting materials for biological weapons construction because they cause diseases with high case fatality rates, have the potential for aerosol transmission, are easily grown in cell culture to high titers, and have an unusually wide host range that encompasses humans and livestock, such as pigs and horses. The recent discovery of new henipaviruses of unknown virulence and spillover potential in bats[1–3] emphasizes the need to understand this emergent group of viruses.

As disease agents posing a severe threat to both agricultural livestock and human health, HeV and NiV are considered "overlap" select agents regulated by both the US Animal and Plant Health Inspection Service and the Centers for Disease Control and Prevention. As is the case for most select agents, approved therapeutics for human henipavirus infections do not exist. Promisingly, however, there have been rapid advances in developing new therapies and in repurposing existing Food and Drug Administration (FDA)-approved drugs for use in henipavirus infection treatment.

HISTORY AND EMERGENCE OF HENIPAVIRUSES

Hendra Virus

Thoroughbred horse racing has been one of the great pastimes of Australia. The public imagination was struck in September 1994 when a prominent racehorse trainer, Vic Rail, 13 of his horses, and another horse from a neighboring property died of a sudden illness in Hendra, Australia. Rail's stablehand also became ill, but recovered after an extended convalescence. The illness in both horses and humans was characterized by respiratory distress with neurological signs, and it culminated in blood-tinged, frothy nasal discharge in the horses.[4,5]

A major outbreak investigation ensued resulting in the identification of a new "equine morbillivirus" from infected horse and human samples. Experimental inoculation of this virus into naïve horses reproduced the disease.[6] Preliminary phylogenetic analysis resulted in the placement of this new agent in the genus *Morbillivirus* (of which measles virus is the prototype member) in the family *Paramyxoviridae*. As this "equine morbillivirus" had unique molecular and pathogenic features that distinguished it from other morbilliviruses, the agent was renamed Hendra virus and finally reclassified in a novel paramyxoviral genus, *Henipavirus*.[7]

A year after the Hendra outbreak, a horse stud owner died of relapsed encephalitis and was retrospectively diagnosed with HeV infection, originating from an encephalitic illness that predated the Hendra outbreak by several weeks. This single infection, which had no known epidemiological link to the Hendra outbreak, occurred 970 km north of Hendra, near Mackay. As in the Hendra outbreak, transmission to the stud owner involved horses, two of which died.[8] Since the 1994 outbreaks, HeV infection emerged only periodically, and briefly, in 1999 and 2004. Then HeV infection emerged yearly from 2006 and proceeded in a truly accelerated fashion from 2011, all in Australia and ranging up and down the east coast (Figure 22-1).[9] All disease outbreaks involving horses had an 84% case fatality rate,[10] and of the seven human cases, including the 1994 outbreaks, four died. Considering that retrospective inspection of laboratory records and historical samples did not reveal signs of pre-1994 spillover of HeV to horses,[11] HeV infection may have emerged from its natural reservoir in or around 1994. The lack of an epidemiological link between the outbreaks[11] suggested potential spillover from another animal reservoir to the horses. However, extensive sampling of domestic animals and wildlife initially revealed no sign of HeV.[11–14] A more targeted investigation based on outbreak characteristics (host should be present in both outbreak locales, be able to move or interchange between the locales, and plausibly have contact with horses) revealed that fruit bats of the genus *Pteropus* (Figure 22-2), otherwise known as "flying foxes," had anti-HeV antibodies[15] and sometimes were infected with HeV itself.[16]

Nipah Virus

The emergence of NiV has several parallels to that of HeV, but with more dramatic consequences. NiV is named after Sungai Nipah, Malaysia, the home village of the patient from whom NiV was first isolated. NiV, like HeV, emerged from pteropid fruit bats, but in this case spilled over to domestic pigs (*Sus scrofa domesti-*

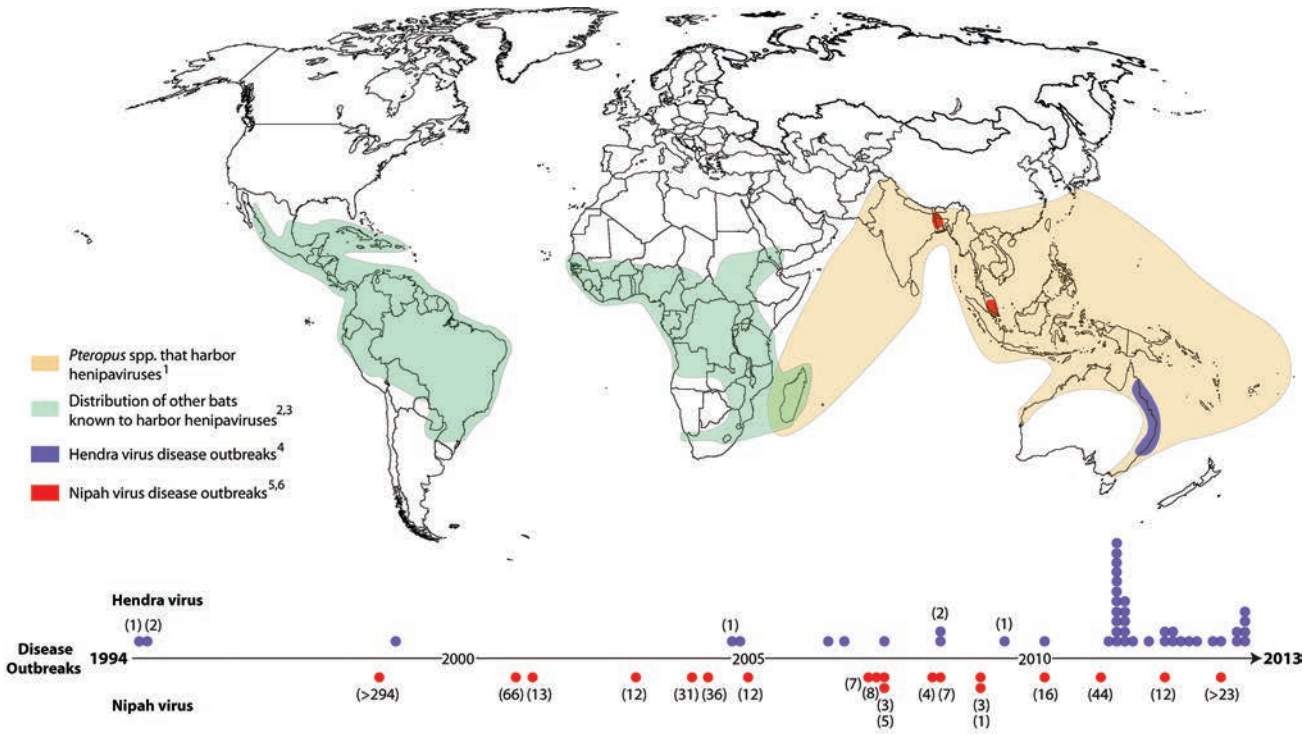


Figure 22-1. The bats that harbor henipaviruses, including Hendra virus and Nipah virus, have widespread distribution. However, outbreaks of Hendra and Nipah disease have been limited to Australia and Malaysia/Bangladesh, respectively. Each outbreak shown on the timeline may represent more than one spillover event from the bat reservoir.
Data sources: (1) Eaton BT, Broder CC, Middleton D, Wang LF. Hendra and Nipah viruses: different and dangerous. *Nat Rev Microbiol*. 2006;4:23–35. (2) Drexler JF, Corman VM, Müller MA, et al. Bats host major mammalian paramyxoviruses. *Nat Commun*. 2012;3:796. (3) Breed AC, Meers J, Sendow I, et al. The distribution of henipaviruses in Southeast Asia and Australasia: is Wallace's line a barrier to Nipah virus? *PLoS One*. 2013;8:e61316. (4) Australian Veterinary Association. *Hendra virus*. http://www.ava.com.au/hendra-virus. Accessed February 13, 2014. (5) World Health Organization Regional Office for South-East Asia. *Nipah virus outbreaks in the WHO South-East Asia Region*. http://www.searo.who.int/entity/emerging_diseases/links/nipah_virus_outbreaks_sear/en/. Accessed February 13, 2014. (6) World Health Organization Regional Office for South-East Asia. *Surveillance and outbreak alert: Nipah virus*. http://www.searo.who.int/entity/emerging_diseases/links/nipah_virus/en/. Accessed February 13, 2014.

cus), which served as highly effective amplifying hosts. The first recorded potential NiV spillover occurred in 1997, with unusual pig deaths at pig farms near Ipoh, Malaysia, attributed to classical swine fever at the time. However, retrospective analysis of serum samples from several patients presenting with encephalitis in 1997 detected anti-NiV antibodies.[17]

Beginning in September 1998, also near Ipoh, pigs and pig farmers became ill in a major outbreak of febrile encephalitis with respiratory symptoms. The mosquitoborne Japanese encephalitis virus (JEV), endemic to Southeast Asia, was immediately suspected, especially since domestic pigs are known to serve as an amplifying host for JEV,[18] and also because 4 of 28 initial human cases tested positive for anti-JEV antibodies.[17] Despite extensive anti-JEV measures, however, including mosquito control and a JEV vaccination campaign,[19] the outbreak continued unabated, spreading into neighboring districts and afflicting increasing numbers of pigs and humans. The outbreak also spread to Singapore via exported infected pigs.[20] Furthermore, the outbreak was widespread with disease mainly afflicting adults,[21] whereas Japanese encephalitis is asymptomatic in the vast majority of cases and more likely to cause disease in children.[18] Also, cerebrospinal fluid from an infected patient gave rise to syncytia (multinucleated cells formed by cell-cell fusion) in Vero cell culture, indicative of a pH-independent fusion mechanism of the infectious agent.[22] Such formation is uncharacteristic of JEV, which has a pH-dependent fusion mechanism. Finally, the Centers for Disease Control and Prevention



Figure 22-2. One of the reservoirs of Hendra virus: a spectacled flying fox (*Pteropus conspicillatus*) near Cairns, Australia. Photograph: Courtesy of Pål A Olsvik, Bergen, Norway.

determined by cross-reactive immunofluorescence, serology, and sequence analysis that the novel agent was closely related to HeV.[23]

At the end of the 1998–1999 outbreak, at least 294 human infections resulting in clinical encephalitis were recorded in Malaysia and Singapore, of which 106 infections were fatal.[17,20] The inclusion of milder nonencephalitic cases (as determined by exposure to infected pigs and seroconversion, a number of which later developed late onset encephalitis) increased the total number of cases to more than 360.[24,25] More than a million pigs were culled in an effort to contain the outbreak, resulting in severe economic damage and widespread changes to the pig farming industry.[26,27] Subsequent outbreaks of NiV infection have occurred in Bangladesh and adjacent areas of India on an almost yearly basis (Figure 22-1). In contrast to the Malaysian outbreak, these subsequent spillovers from pteropid bats have mainly occurred without involvement of a domestic animal intermediate. Generally these spillovers had higher case fatality rates, ranging from 40% to 100%.[28] These differences may be the result of differences among disparate NiV strains, the standard of care in Bangladesh/India versus Malaysia, or



Figure 22-3. (**a**) In Bangladesh, fresh date palm sap is collected by shaving the date palm tree, placing a tap, and collecting the sap in a clay pot overnight. Fruit bats are known to lick the sap stream and even urinate or defecate into the clay pots, thus potentially contaminating the raw sap with infectious Nipah virus. (**b**) Covering the sap stream with a bamboo skirt is highly effective at preventing contamination.
Photographs: Courtesy of Nazmun Nahar, International Centre for Diarrhoeal Disease Research, Bangladesh.

sociocultural factors unique to Bangladesh/India.[29,30] For example, a major route of spillover during the Bangladesh outbreaks is the consumption of fresh date palm sap, a seasonal delicacy that can become contaminated with fruit bat saliva or excreta during collection (Figure 22-3).[31]

Newly Identified Henipaviruses

After nearly two decades of intensive investigation of HeV and NiV, evidence of novel henipaviruses in diverse wildlife species has created increased public health concern.[1–3,32–36] For example, the recent discovery of divergent new clades of henipaviruses in indigenous bat populations across Africa and even Central/South America,[1,34] at least another henipavirus endemic to Australia but distinct from HeV (Cedar virus),[32] and a henipavirus in southeastern China (Mòjiāng virus) from a putative rodent reservoir that may be linked to cases of fatal pneumonia in humans,[35] all raise urgent questions about the possible global emergence of these zoonotic viruses. These new findings extended the potential geographic distribution of henipaviruses from Southeast Asia/Australia to Africa and Central/South America (Figure 22-1). At least one of these novel henipaviruses, Cedar virus, could be isolated in culture, but was nonpathogenic in small animal models.[32] The discovery of a potential henipavirus spillover to humans in Cameroon, however, emphasizes the need to both increase vigilance and knowledge of this latent pool of henipaviruses.[3] Human sera from Cameroon capable of neutralizing NiV were significantly associated with bat butchering in areas of intensive deforestation.[3] This association highlights the role of environmental changes and specific human behaviors in determining the risk of zoonotic transmission. Although the pathogenicity and virulence of newly discovered henipaviruses remain to be determined, the repeated misdiagnosis of NiV as Japanese encephalitis in Southeast Asia remains a cautionary tale. Henipavirus-derived illness may often be ascribed to other encephalitic diseases known to occur in the affected area.

EPIZOOTIOLOGY AND EPIDEMIOLOGY OF HENIPAVIRUSES

Pteropid (fruit) bats appear to be the major reservoir host for HeV and NiV. No appreciable signs of HeV and NiV infections have been found in other wildlife or domestic animals.[11,37] Anti-HeV or anti-NiV antibodies are highly prevalent in pteropid bat populations throughout Southeast Asia and Australia[37,38]; HeV and NiV have been isolated from *Pteropus* fruit bats throughout their geographic range[37]; and all known outbreaks of HeV and NiV infection are linked to exposure of domestic animals or humans to fruit bats or their excretions.[30] Furthermore, experimental infection of *Pteropus* bats belonging to different species with HeV or NiV did not result in clinical signs of infection, despite inconsistent signs of seroconversion, viral replication in tissues, and virion excretion in urine.[39–42] These data support the hypothesis that HeV and NiV are not pathogenic in their natural *Pteropus* hosts, thus persisting subclinically in these bat populations.

Despite their apparent long coevolutionary history with bats and likely endemic nature,[37] HeV and NiV emerged to cause human disease suddenly and nearly simultaneously, likely signaling common factors driving their emergence. A retrospective study of environmental factors and man-driven changes in Malaysia identified slash-and-burn agriculture with concomitant loss of forest habitat, a resulting impenetrable smoke haze that led to crop failures, and severe drought as potential precipitating factors.[43] Fruit bats were driven to populate cultivated fruit orchards, which were often located adjacent to pig farms, thus providing a means of transmission. In one plausible scenario, half-eaten fruits contaminated with NiV-infected bat saliva are dropped into a pig farm and then eaten by the pigs. Such half-eaten fruits were found in pig farms near the epicenter of the 1998–1999 NiV encephalitis outbreak.[43] Similar environmental and manmade pressures facing fruit bat populations across Southeast Asia and Australia are likely behind the persistent upsurge in HeV and NiV spillovers in recent years.[4]

Hendra Virus

All seven known human cases of HeV infection resulted from intimate contact with sick horses. HeV is highly virulent in horses, and infection often culminates in copious production of infectious respiratory secretions.[38] Human infections were traced back to efforts to save these horses without the use of personal protective equipment. A horse trainer, for example, attempted to force-feed a sick mare with abrased bare hands.[5] However, such cases of horse-to-human transmission remain the exception. Many other people who were also highly exposed to contaminated horse bodily fluids, even to fluids from horses that were implicated in HeV transmission to other humans, did not develop signs of HeV infection.[30] With increased public awareness of the risk of HeV transmission and the corresponding increase in proper use of personal

protective equipment, the risk of spillover to humans has been mitigated.[4] The introduction of an HeV vaccine for horses in 2012, Equivac HeV (Pfizer Animal Health, Brisbane, Australia), will hopefully further minimize human transmission risk.

How horses initially become infected remains to be elucidated. Equine cases of infection may result from horses grazing on pastures contaminated with bat excreta or remains of half-eaten fruit.[11,37] Infected horses inefficiently transmit the virus, even to other horses, but some horse-to-horse transmission may occur through licking infectious nasal discharge.[11] Respiratory transmission of HeV has never been demonstrated experimentally or during natural infection.[37] The inefficiency of HeV transmission is buttressed by findings that despite a continuous low prevalence of HeV in *Pteropus* bats in Queensland and New South Wales,[44] people having extensive contact with *Pteropus* bats in these areas, with bat bites and exposures to bat blood in many cases, had no sign of HeV exposure.[45] Direct comparisons of NiV and HeV infection in some rodent models suggest that HeV may be less efficient in infecting animals through the intranasal route,[46,47] although this lower efficiency remains to be verified in a more relevant nonhuman primate model.

Nipah Virus

Malaysia Outbreak, 1998–1999

During the large Malaysia outbreak, pigs served as a highly effective amplifying reservoir, contracting infection and transmitting NiV so efficiently that the infection rate among pigs at affected farms approached 100%.[27] The risk to humans posed by this spread was exacerbated by the mild illness NiV caused in the pigs, with a lethality of less than 1% to 5% and often presenting asymptomatically.[27] Unsuspecting farmers thus moved asymptomatic pigs to other farms and slaughterhouses, quickly spreading the virus. A subset of infected pigs developed febrile illness, respiratory signs such as labored breathing and harsh nonproductive cough, and neurological signs such as myocloni and uncoordinated gait.[27]

Nearly all human cases during the Malaysia NiV encephalitis outbreak can be attributed to direct contact with or proximity to infected pigs, and most cases were pig farmers.[30,48,49] One person who denied any recent proximity to pig farms repaired pig cages, suggesting that the virion-containing secretions remained infectious on surfaces for extended periods.[50] A few human infections may have resulted from secondary transmission through dogs, which were commonly infected during the outbreak.[11,49,51] Evidence of human-to-human transmission is limited. A large cohort study of 393 healthcare workers intimately involved in caring for NiV-infected patients identified only three nurses who seroconverted and had potential illness, despite many reported high-risk exposures.[52] Risk may have been minimized by precautions taken by the healthcare workers, because patients clearly shed infectious NiV in respiratory secretions and urine, especially during the early phase of illness.[53] Human-to-human transmission possibly may have played a role in the NiV outbreak, although the epidemiological record does not provide unambiguous evidence.[30] Finally, retrospective investigations appear to show that NiV was causing disease in pigs as early as 1996, but that the mild symptoms and rough similarity to other diseases (eg, classical swine fever) did not raise suspicion of anything unusual.[11,54] The advent of increasingly inexpensive, high-throughput sequencing may make disease surveillance and agent identification more likely to catch emerging pathogens such as NiV in the future.

Bangladesh and India, 2001 to Present

In comparison to the Malaysia NiV encephalitis outbreak, subsequent outbreaks in Bangladesh and India are of greater concern because of evidence of clear chains of human-to-human transmission.[30] As in Malaysia, infected patients shed NiV in their bodily fluids including saliva.[55,56] The intimate care for sick family members, involving being in physical contact, sharing utensils and food, and sleeping in the same bed to provide them comfort, greatly increased the risk of transmission.[57] The longest documented transmission chain involved five generations, with the third generation involving a religious leader who infected 22 family members and followers.[57,58] A major 2001 outbreak in Siliguri, India, was also characterized by hospital-associated transmission: one admitted patient became the source of more than 40 subsequent infections within a hospital and nursing homes. [59] As with the outbreak in Malaysia, Japanese encephalitis was initially suspected, and the causative agent was not identified as NiV until samples were retrospectively analyzed several years later.[59]

The outbreaks in Bangladesh and India are also notable for the apparent lack of a domestic animal intermediate between the *Pteropus* bat reservoir and humans. Although a few incidents appear to involve domestic animals such as cows, pigs, or goats,[60–62] the major route of spillover has been the consumption of contaminated fresh date palm sap.[31] Date palm trees are tapped for their sweet sap in the winter, and bats often lick the sap stream. Defecation and urination

into or near the collection pots, or even drowned dead bats, have been observed.[31,63] Fortunately, the use of a simple bamboo skirt to cover and protect the sap stream, a method local to northwest Bangladesh that has not been consistently or widely used, appears to be highly effective in preventing contamination (Figure 22-3).[64] More widespread use of this method and other interventions to minimize risk would help the affected regions of the Indian subcontinent break out of its cycle of yearly NiV encephalitis outbreaks.

CLINICAL PRESENTATION OF HENIPAVIRUS INFECTIONS

The incubation period of HeV or NiV infection and illness typically ranges from a few days to 14 days.[65] Although information on HeV infection is limited because of the few human cases, the clinical signs and pathology of HeV and NiV infections are similar.[66] The hallmarks of henipavirus pathogenesis are extensive vasculopathy, respiratory distress, and encephalitic disease with corresponding neurological symptoms. Respiratory and encephalitic symptoms may appear to varying degrees. During the first Malaysia NiV disease outbreak, clinical signs were mainly encephalitic in nature with minor pulmonary involvement, whereas in subsequent outbreaks in Bangladesh, encephalitis was more commonly joined by severe respiratory distress.[29,62] Researchers are actively investigating whether these differences result from genetic differences among viral variants, the route of transmission, or other factors.

Clinical Signs and Symptoms

Neurological signs and symptoms may include fever, headache, confusion, myocloni, seizures, meningism, and motor deficits including areflexia and hypotonia.[5,29,48,59,67,68] Brain stem involvement, a poor prognostic factor, may be evidenced by a reduced level of consciousness, vomiting, abnormal pupillary and doll's eye reflex, hypertension, and tachycardia.[48]

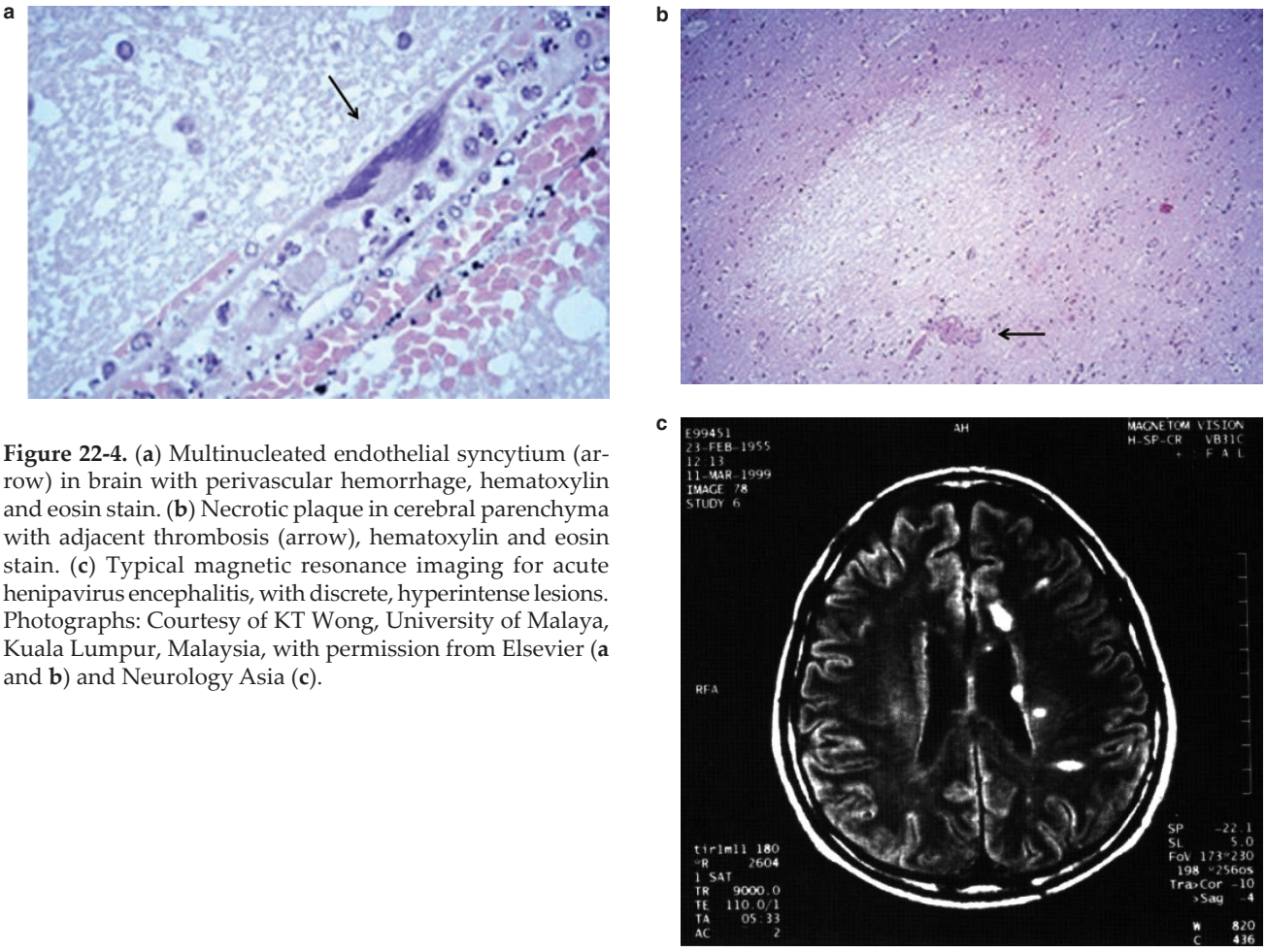


Figure 22-4. (**a**) Multinucleated endothelial syncytium (arrow) in brain with perivascular hemorrhage, hematoxylin and eosin stain. (**b**) Necrotic plaque in cerebral parenchyma with adjacent thrombosis (arrow), hematoxylin and eosin stain. (**c**) Typical magnetic resonance imaging for acute henipavirus encephalitis, with discrete, hyperintense lesions. Photographs: Courtesy of KT Wong, University of Malaya, Kuala Lumpur, Malaysia, with permission from Elsevier (**a** and **b**) and Neurology Asia (**c**).

Cerebrospinal fluid is characterized by elevated white blood cell counts and/or protein concentration in a substantial proportion of cases.[48,69]

A minority of survivors (<10%) of HeV and NiV infection may experience relapsing encephalitis after apparent recovery or even initial asymptomatic or apparently nonencephalitic infection. Relapse may occur soon after apparent recovery or long after, with an average of 8 months and up to 11 years documented.[65] Even without relapsing or progressive disease, a substantial proportion of survivors may experience long-term neurological deficits.[70,71]

Although the Malaysia outbreak of NiV disease was mainly characterized by encephalitic signs, a substantial proportion of patients still developed pulmonary signs such as cough and abnormal chest radiographs.[20,48,68] More severe pulmonary symptoms, seen in subsequent NiV disease outbreaks and also some cases of HeV infection (eg, a horse trainer who developed progressive respiratory failure[5]), may also include atypical pneumonia, breathing difficulty, and acute respiratory distress syndrome.[29,65]

Pathology

Infection of microvascular endothelial cells leads to systemic vasculitis, thrombosis, and resultant microinfarction, especially in major organs such as kidneys, heart, lungs, and brain.[65,72] Focal perivascular necrosis or hemorrhage is seen in highly vascular organs such as the spleen.[73] Syncytia (giant multinucleated cell) formation is occasionally seen in the endothelium (Figure 22-4a) and among parenchymal cells of major organs. In the brain, discrete plaque-like lesions with varying degrees of necrosis, edema, and inflammation (Figure 22-4b) likely correspond to the small hyperintense lesions in both grey and white matter seen by magnetic resonance (Figure 22-4c).[65] The dual pathology of vasculitis with associated microinfarction and direct infection of parenchyma of major organs is a distinguishing feature of henipavirus pathogenesis.[65] Tissue damage in the central nervous system from both microinfarction and direct infection of neuronal cells distinguishes henipavirus infections from other viral encephalitic diseases.[74] Relapse encephalitis appears to result from recrudescent infection, with extensive parenchymal necrosis, edema, and inflammation corresponding to confluent lesions seen in magnetic resonance scans (as opposed to the more discrete foci usually seen during acute encephalitis during early illness). Pathology associated with relapsing encephalitis is only found in the central nervous system, and no vasculopathy is present, even in the brain.[25,75]

MOLECULAR BIOLOGY OF HENIPAVIRUSES

Virus Structure

Henipaviruses are negative-sense ribonucleic acid (RNA) viruses that produce enveloped virions. Henipaviruses are currently classified as members of the genus *Henipavirus*, family *Paramyxoviridae*, order *Mononegavirales*.

Like other paramyxovirus particles, Hendra and Nipah virions have pleomorphic shapes, usually more spherical although sometimes filamentous as well (Figure 22-5a), and range in size from less than 200 nm to more than 1,000 nm in diameter.[76,77] The virions contain helical ribonucleocapsids with the herringbone appearance characteristic of, and unique to, paramyxoviruses (Figure 22-5b). A unique feature of Hendra virions is the frequent presence of a double fringe surrounding the particle,[77] which may result from differing lengths or conformations of the envelope proteins; in contrast, Nipah virions predominantly display a single fringe.

Virus Genome

Like all mononegaviruses, henipaviruses have a linear, monopartite, single-stranded RNA genome of negative polarity. The overall structure of henipavirus genomes is similar to those of other paramyxoviruses, with 3′ leader and 5′ trailer sequences at the termini of the genomes that act as virus-specific promoters, 5′ and 3′ untranslated regions flanking each gene, and a conserved intergenic signal between each gene.[78] With a few recently described exceptions, henipaviruses have the longest known paramyxoviral genomes (≈18 kb). The especially long 3′ untranslated regions are unique features of henipavirus genomes and account for much of the extra length compared to other paramyxoviruses (≈15 kb).[79] The functional relevance of these long untranslated regions remains to be determined.

Viral Proteins

Henipavirus genomes contain six genes, which encode a nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), fusion protein (F), attachment glycoprotein (G), and large RNA-dependent RNA polymerase (L). N encapsulates the genome, thereby forming the ribonucleoprotein complex. Like the genomes of other paramyxoviruses, henipavirus genomes have nucleotide lengths that are evenly divisible by six, a feature likely resulting from the periodicity of N protein encapsidation of

the genome. P and L form the polymerase complex, which replicates the viral genome and transcribes viral mRNAs.

M organizes the assembly and budding of virions and underlies the viral envelope. M also transits through the nucleus, although the ultimate significance of this transit for henipavirus replication and pathogenesis remains unclear. G binds to the cellular receptor(s), and F catalyzes the membrane-membrane fusion responsible for viral entry. G and F can also catalyze cell-cell fusion, leading to syncytium (giant multinucleated cell) formation, which is a hallmark of henipavirus infection (Figure 22-5c). The henipavirus attachment protein is unique among paramyxoviruses in that it does not possess any hemagglutinin activity (although morbilliviruses such as measles virus will only agglutinate red blood cells from primates of certain species).[80]

The tissue tropism of HeV and NiV is determined by entry receptor use. The receptor tyrosine kinase ephrin-B2 serves as an entry receptor for all known henipaviruses,[32,81,82] but at least HeV and NiV can also use the related ephrin-B3 as an alternative receptor.[83,84] As cellular factors that are critical during embryogenesis, ephrin-B2 and -B3 are highly evolutionarily conserved. This high level of conservation contributes to the unusually wide potential host range of HeV and NiV, which have been shown to infect mammals spanning six orders.[85] Ephrin-B2 and -B3 from a wide range of mammals allow viral entry,[86] chicken embryos are susceptible to NiV infection,[87] and even zebrafish ephrin-B2 can serve as an entry receptor.[72] The tropism of henipaviruses is also not restricted by the cellular protease required for F protein cleavage, a maturation step required to render it fusion-competent. Unlike some paramyxoviral F proteins that require a protease of limited tissue distribution, the henipavirus F protein uses the ubiquitously expressed endosomal protease cathepsin L,[88,89] thereby further extending tissue and possibly host tropism.

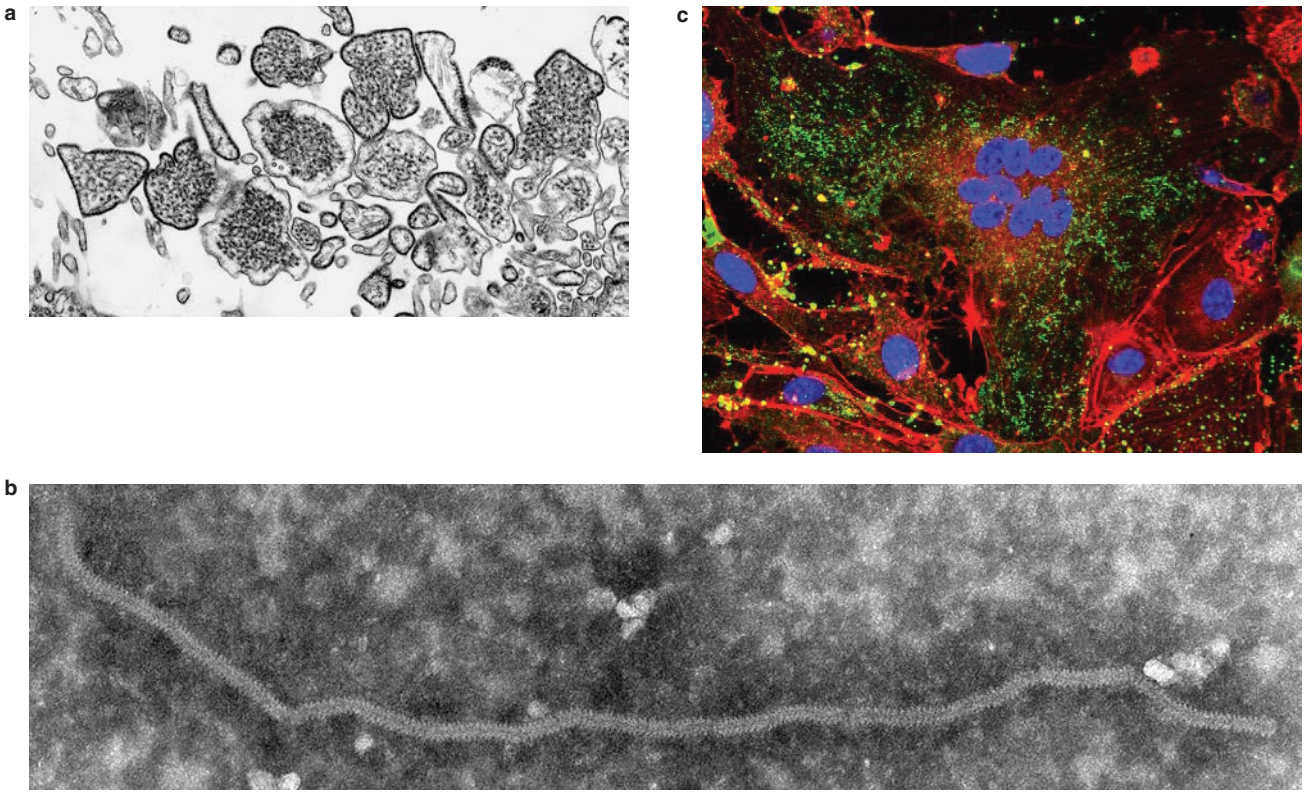


Figure 22-5. (**a**) Nipah virions produced from infected Vero E6 cells. (**b**) Nipah virus nucleocapsid, with the herringbone appearance characteristic of paramyxoviruses. (**c**) Multinucleated Nipah virus-induced syncytium in human umbilical vein endothelial cells. Blue represents nuclei; red represents actin filaments and illustrates cell boundaries; and green represents Nipah virus matrix protein.
Photographs: (**a** and **b**) Courtesy of Cynthia Goldsmith, Centers for Disease Control and Prevention, Atlanta, Georgia. (**c**) Courtesy of Arnold Park, Icahn School of Medicine at Mount Sinai, New York, New York.

PATHOGENESIS

The pathogenesis of henipavirus encephalitis has been examined predominantly using animal models. The oronasal route of infection is used most often during such experiments as it reflects at least one type of natural transmission and might also simulate exposure during a biological attack.[90] Similar to results obtained in vitro, in vivo tropism is predominantly determined by the absence or presence of henipavirus receptors on potential target cells.

Ephrin-B2 is highly expressed on the endothelial and smooth muscle cells that line arterial vessels, lungs, and brain. The human airway epithelium expresses both ephrin-B2 and -B3,[91] and upon infection, the virus likely crosses the epithelium via limited basolateral virion release, while disruption of the epithelium via cell-cell fusion may also facilitate systemic entry. In many animal models of Nipah encephalitis, the alveolar epithelium tests immunopositive for NiV antigens, and the alveolar wall frequently undergoes fibrinoid necrosis.[92] Interestingly, in pigs, an increased number of alveolar macrophages is a consistent histological feature. In human lung tissue, viral antigen is found in multinucleated giant cells located in the alveolar space.[73] Multinucleated giant cell formation is a product not only of alveolar macrophage fusion, which is generally a prominent feature of chronic inflammatory conditions, but also of NiV-induced syncytia formation. Transmigration of infected alveolar macrophages may serve as a "Trojan horse" for the virus to gain systemic access via the microvasculature. Cell-free and cell-associated viremia then result in systemic infection.[93]

Ephrin-B3 expression is mostly restricted to the central nervous system (eg, brainstem), which correlates with the observation of antemortal brainstem dysfunction noted during henipavirus encephalitis.[48,83] Henipaviruses likely gain access to the brain via basolateral release from brain microvascular endothelial cells, disruption of the blood-brain barrier as a result of cell–cell fusion and vasculitis, and transmigration of "Trojan horse" inflammatory macrophages. It is also possible that henipaviruses access the central nervous system more directly via infection of the olfactory epithelium and spread along the olfactory neurons.[94,95]

Clear differences exist between HeV and NiV infections regarding localization of initial infection in animal models despite their use of the same entry receptors,[46,47] which has yet to be understood. The mechanism of foodborne NiV transmission also is unclear since digestive tract epithelium does not express ephrin-B2 or -B3.[72] Virus infection most likely occurs via accessible and susceptible cells in the oropharyngeal mucosa, such as those of tonsillar tissues and salivary glands.

Henipaviruses partly owe their virulence to effective antagonism of host immune responses. Interferons (messengers of innate immunity that trigger extensive antiviral responses) bind extracellularly to interferon receptors, which usually trigger an intracellular signal cascade that includes the critical immune signaling factor signal transducer and activator of transcription 1 (STAT1). Like other paramyxoviruses, HeV and NiV encode several nonstructural proteins from their P genes, that is, the C, V, and W proteins, which antagonize innate immune signaling. The P, V, and W proteins have identical N-termini that bind and inhibit STAT1, partly by sequestering STAT1 and thus inhibiting transcription of interferon-inducible genes.[96–99] The P gene products also antagonize signaling from other intracellular sensors of infection, such as TLR3 and Mda-5,[100–102] which sense double-stranded RNA (an intermediate of viral replication) in endosomes and the cytoplasm, respectively.

In contrast to the host species-specific inhibition of interferon signaling induced by some paramyxoviruses,[103] NiV can inhibit interferon signaling in cells from a number of tested mammals,[104] consistent with the ability to cause disease in a wide range of hosts. The high virulence of henipaviruses in the "wrong" host may therefore be a function of their broad tissue tropism in vivo, their ability to gain systemic access, and their inhibitory effect on immune responses.

HENIPAVIRUSES AND BIOLOGICAL WEAPONS

HeV and NiV are classified as Centers for Disease Control and Prevention Category C Bioterrorism agents because of the following:

- their availability,
- ease of production and dissemination, and
- potential for high morbidity and lethality.

Availability

HeV and NiV are readily available from tissues or fluids from patients during frequent, recurring disease outbreaks as well as by isolation from their natural bat reservoirs. Furthermore, reverse genetics systems are available in biosafety level 4 laboratories for the rescue

of henipaviruses directly from plasmids.[105,106] De novo access to, and modification of, henipaviruses can be accomplished by a determined hostile group with access to resources and the relevant technical expertise.

Ease of Production and Dissemination

Henipaviruses can be grown to very high titers, up to 10^8 $TCID_{50}$/mL or PFU/mL,[107] in a wide range of cell lines.[108] Although henipaviruses are highly sensitive to temperature variation and dessication, henipaviruses can persist for days under certain conditions.[109] Therefore, under optimal conditions henipaviruses could possibly be maintained at high titer for extended periods of time. Henipaviruses infect a wide range of hosts, including domestic animals such as dogs and livestock, that could serve as amplifiers of infection. Although NiV and HeV clearly have the potential to infect through aerosol, this route has not been conclusively shown, either experimentally or from the natural history[90,110]; however, limited epidemiological evidence suggests that some human cases of NiV infection in Bangladesh may have resulted from exposure to coughing.[58,111] Also restricting the biological weapons potential of henipaviruses is the lack of sustained human-to-human transmission: the longest documented chain of transmission was five generations.[57,58] A naturally occurring or intentionally mutated strain with higher transmission efficiency would be required to sustain an epidemic; an expanding pandemic, however, may not be a required or desired goal of a bioterror attack.

Potential for High Morbidity and Lethality

HeV and NiV cause disease with very high case fatality rates, ranging from 40% to 100% in recent outbreaks.[28] Survivors of disease or asymptomatically infected people may present years later with relapsing or late onset encephalitis, indicating occasional persistence of viral infection.[65]

DIAGNOSIS

In a bioweapons attack or other mass casualty scenario, rapid diagnostic methods must be used to identify the causative agent(s). These methods may be differentiated by whether or not the specific agent must be suspected before testing and by how quickly the results can be obtained. Under normal circumstances, henipavirus etiology would not be suspected without exposure to risk factors (contact with bats, ill persons, or domestic animals, or consumption of raw date palm sap) in the currently affected areas of Southeast Asia and Australia. Henipavirus etiology may also be considered throughout the known geographic range of bats known to harbor henipaviruses (Figure 22-1) if the responsible agent is unknown, with the initial misdiagnosis of NiV as Japanese encephalitis virus remaining a cautionary tale. These factors do not apply in a bioweapons or bioterrorism event, however. The clinical presentation of henipaviruses with encephalitic and/or respiratory symptoms cannot be readily distinguished from other viral and nonviral causes of encephalitis, and diagnosis requires epidemiological and laboratory investigation. Given the broad host range of henipaviruses, the involvement of sick domestic or local animals can suggest potential henipavirus etiology.

Detailed recent reviews of henipavirus diagnosis may be found elsewhere[107,112,113]; the available methods are discussed below.

Reverse Transcription-Polymerase Chain Reaction

If henipaviruses are suspected, henipavirus-specific polymerase chain reaction or real-time polymerase chain reaction should be performed on RNA extracted from patient samples. Prospective samples include serum, whole blood (detectable viremia may be cell-associated[93]), urine, nasopharyngeal aspirates, throat swabs, cerebrospinal fluid, or tissue samples from highly affected tissues such as the brain, lungs, kidneys, or spleen.

Detection of Henipavirus Antigens

Characterized anti-henipavirus antibodies can be used to detect viral antigens in formalin-fixed tissues. Similarly, immunofluorescence with anti-henipavirus antibodies can be performed on infected cell cultures.

Detection of Anti-henipavirus Immune Responses

A number of methods exist for detection of anti-henipavirus antibodies in sera of infected patients. Specific immunoglobulin M responses develop in virtually all patients within the first week postexposure, and the slower immunoglobulin G response encompasses virtually all patients after 2 weeks of infection.[29,114] Detection of serum antibodies is useful for diagnostics because antibodies are more stable than viral RNA over time and under different conditions. Furthermore, viremia

can be difficult to detect when patients are symptomatic. Serum neutralization of replicating virus is considered the gold standard serological test, although this test requires biological safety level 4 containment.[107] Surrogate neutralization tests, which can be performed at biological safety level 2 conditions, offer a combination of high sensitivity and high specificity. Such tests include the use of vesicular stomatitis Indiana virus pseudotyped with the henipavirus envelope proteins[115–117] or a Luminex (Luminex Corporation, Austin, TX) platform-based assay assessing the ability of sera to inhibit the binding of the henipavirus receptor to microbeads coated with henipavirus attachment protein.[118] Enzyme-linked immunosorbent assay variations on this test using infected cell lysate or recombinant henipavirus proteins have been commonly used as frontline assays because of their simplicity and affordability, but typically have a relatively high false-positive rate.[107]

Virus Isolation

Viral etiology may be suspected if cells incubated with filtered patient samples develop cytopathic effects. Henipaviruses grow efficiently in a wide range of cell lines, including Vero E6 cells.[119] Syncytia formation in cell culture would implicate enveloped viruses with a pH-independent fusion mechanism, which include henipaviruses (Figure 22-5c). Electron microscopy on viral preparations and infected cells could implicate a henipavirus as the potential agent. New or modified henipaviruses may have different characteristics, however, so any final diagnosis requires multiple routes of confirmation. In contrast to sequencing, virus isolation is a days-long process (2–5 days usually pass until cytopathic effects become visible), and two rounds of 5 days each are recommended before virus recovery is judged unsuccessful.[107,112] Furthermore, for an outbreak suspected to be caused by a highly virulent agent, virus isolation attempt should be performed under high-level biosafety containment. Nevertheless, isolation and characterization of the agent remain the most conclusive demonstration of etiology.

Next-generation Sequencing

Next-generation sequencing (NGS) is slowly becoming more widely available and will enable the identification of known and unknown henipaviruses. As the cost and speed of NGS continue to decrease, a rapid and routine measure using NGS in a suspected outbreak is increasingly possible following RNA extraction and reverse transcription polymerase chain reaction.[107] Since henipaviruses only rarely infect humans, detection of henipavirus sequences would not normally be expected, and an appreciable presence of henipavirus sequence in multiple samples would suggest potential henipavirus etiology. Henipavirus-specific NGS is already becoming routinely used during postoutbreak investigations to determine the characteristics of new henipavirus isolates.[107,120]

MEDICAL MANAGEMENT

Licensed therapeutics to treat henipavirus infection in humans are not available. Thus, medical management of henipavirus infections is supportive. Mechanical ventilation is required if the patient becomes comatose or develops acute respiratory distress syndrome. Appropriate measures should be taken as for any potentially highly contagious pathogen, including quarantine and use of personal protective equipment and engineering controls such as negative air flow, if available; careful handling of clinical specimens; and rapid epidemiological investigation (with particular attention to potential spread via domestic animals) and identification of high-risk contacts. Despite the lack of specifically recommended therapeutics, several potential treatments with varying levels of supporting evidence should be considered in the event of a bioweapons or mass casualty event. Some of the proposed therapeutic interventions discussed below may also be appropriate in the case of accidental exposure or as prophylaxis for frontline responders to a potential outbreak.

Passive Immunotherapy

Active vaccination is highly effective in animal models[83] and was the basis for the recently approved Equivac HeV vaccine for horses. This vaccine contains a soluble version of the HeV attachment envelope protein, which stimulates the production of neutralizing anti-HeV antibodies and provides protection against HeV infection.[121] However, an active vaccination approach for henipaviruses is unlikely to be a practical strategy on a population-wide basis in humans for several reasons. Compared to veterinary vaccines, human vaccines have higher regulatory hurdles. In addition, such vaccines would be truly useful only for a few people. Few cases have occurred during natural outbreaks, and the likelihood that populations outside of affected locations will be exposed to a pathogenic henipavirus is low. However, the risk–benefit calculations may be different for frontline responders to suspected outbreaks.

Passive immunotherapy may be highly efficacious as postexposure treatment. In recent postexposure prophylaxis studies, ferrets and grivets received 1 to 2 doses of a human monoclonal antibody with neutralizing activity against HeV and NiV attachment proteins 10 to 72 hours after virus exposure, which protected the animals from disease.[122,123] The antibody has been offered to individuals with high risk of HeV exposure, and human clinical trials started in May 2015 in Australia.[124,125] Although further development of this strategy may not be economically viable if left to the market, such monoclonal antibodies warrant serious consideration as a stockpiled resource that can be used in a limited outbreak or bioweapons attack.

Ribavirin

Ribavirin, a guanosine analog first synthesized in 1970, has been known to have broad-spectrum activity against many RNA and DNA viruses. Ribavirin is mainly used against human respiratory syncytial virus infections and to treat persistent hepatitis C virus infections.[126,127] Ribavirin has several potential antiviral properties, which may differ in importance for different viruses.[128]

Because of its broad-spectrum effect, ribavirin was used in an off-label, nonrandomized, unblinded trial during the first Malaysia outbreak of NiV encephalitis, despite the known adverse effects (primarily hemolytic anemia at high dose).[129] The lethality in the treated group was reduced by 40%, without affecting the rate of anemia. Follow-up studies revealed that ribavirin inhibits HeV and NiV replication in vitro.[67,130–133] Results from in vivo studies examining the efficacy of ribavirin in the hamster[130,131] and grivet[134] animal models consistently found that ribavirin extends time to death. These animal models may represent particularly susceptible models for HeV and NiV pathogenesis because of the reproducibility of human disease.[90] One concern has been that ribavirin only inefficiently crosses the blood–brain barrier, which is particularly consequential for an encephalitic disease. Modifications to the drug administration method, however, have the potential to overcome this hurdle.[135–138]

Current treatment of chronic hepatitis C involves the combination of type I interferon (discussed further in the next section) and ribavirin, which act synergistically.[139] Ribavirin may therefore be evaluated in combination with other promising therapeutics in the case of henipavirus infection.

Innate Immunotherapy

Henipavirus inhibition of cellular production of interferon and of cellular responses to exogenous interferon is incomplete.[140,141] Therefore, a clear opportunity is available to inhibit HeV and NiV pathogenesis by augmenting the innate immune response in vivo. The investigational double-stranded RNA compound poly(I)-poly(C_{12}U), which stimulates type I interferon production, is highly effective against NiV infection in the hamster model when administered immediately after exposure.[131] However, follow-up studies need to address the postexposure therapeutic window. Furthermore, despite undergoing phase III clinical trials for treatment of chronic fatigue syndrome, poly(I)-poly(C_{12}U) (Rintatolimod, Hemispherx Biopharma, Philadelphia, PA) has not been FDA approved.

Recombinant and modified type I interferons (eg, PEGylated interferon-α), however, have been approved by the FDA for multiple uses, including the treatment of chronic hepatitis B and C. Exogenous interferon inhibits henipavirus replication in vitro[141] and would be a more direct approach to treat henipavirus infections than stimulating interferon production with compounds like poly(I)-poly(C_{12}U). However, these compounds have yet to be evaluated against henipavirus infections.

"Off-the-Shelf" Therapies Evaluated In Vitro

Numerous potential therapies to treat henipavirus infections are in varying stages of development (reviewed elsewhere).[142,143] Promising therapies that are already available for off-label use against henipaviruses, but have not been evaluated for efficacy in vivo, are briefly described below.

As NiV M protein requires ubiquitinylation as part of its intracellular trafficking pathway, proteasome inhibitors, which deplete the intracellular pool of free ubiquitin, are potent inhibitors of NiV replication in vitro.[144] Next-generation proteasome inhibitors with improved pharmacokinetics, such as the FDA-approved carfilzomib or orally bioavailable analogs such as oprozomib (currently in phase I/II oncology trials), have even greater efficacy against henipaviruses in vitro.[145] If these effects on henipaviruses can be translated into in vivo potency, the possibility of using potential FDA-approved proteasome inhibitors for off-label use will be a significant and realistic option for exposed or infected frontline responders.

Many clinically available drugs inhibit henipavirus replication in vitro in the low micromolar range, including the alpha blocker phenoxybenzamine as well as the beta blocker propranolol, the antifungal clotrimazole, and the estrogen receptor antagonist tamoxifen.[146] However, it is unclear whether any of these therapies can achieve viral inhibitory concentrations in vivo.

SUMMARY

The recent emergence, high virulence, and pandemic potential of HeV and NiV have fueled public concern and even lead to repeated public calls in Australia for culling of the reservoir hosts, the flying foxes. The worldwide discovery of numerous henipaviruses of unknown pathogenicity, including evidence of a potential spillover event to humans in Africa, further emphasizes the need for continued investigations into all aspects of henipavirus ecology, molecular biology, and pathogenesis. Although HeV and NiV do not appear efficiently transmissible at this time, vigilance for variants (or other henipaviruses) with enhanced transmissibility should be maintained. Significant progress in the development and identification of effective therapeutics for henipaviruses will mitigate the risks involved in managing future outbreaks.

Acknowledgments

We are grateful for the technical writing services offered by Laura Bollinger (IRF-Frederick). The content of this publication does not necessarily reflect the views or policies of the US Department of the Army, the US Department of Defense, the US Department of Health and Human Services, or of the institutions and companies affiliated with the authors. JHK performed this work as an employee of Tunnell Government Services, Inc, a subcontractor to Battelle Memorial Institute under its prime contract with NIAID, under Contract No. HHSN272200700016I.

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Chapter 23

FILOVIRUSES

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INTRODUCTION

NOMENCLATURE
- **Filovirus Taxonomy**
- **Filovirus Disease Nomenclature**
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INTRODUCTION

Human viral hemorrhagic fevers are typically caused by members of the four families: (1) *Arenaviridae* (several mammarenaviruses), (2) *Bunyaviridae* (several hantaviruses, nairoviruses, phleboviruses), (3) *Filoviridae* (certain ebolaviruses, marburgviruses), and (4) *Flaviviridae* (several flaviviruses *sensu stricto*).[1] The viruses of these four families are distinct in their molecular biology, reservoir host spectrum, and transmission route. However, the human diseases these viruses cause are clinically and pathologically similar, and all of the diseases are associated with significant lethalities (case fatality rates).[1] Among these viruses, filoviruses are arguably the most notorious as they are associated with the highest lethality and receive the widest attention in the media.[2] Importantly, filoviruses were included in the Soviet biological weapons research program.[3] The actual achievements of this program are still under debate, but through their inclusion, filoviruses gained military significance. This chapter provides an overview of the diversity, epidemiology, and molecular biology of filoviruses; summarizes the clinical presentation and pathology of the human diseases they cause; and reviews current developments in prophylactics and antivirals for the prevention and treatment of infections.

NOMENCLATURE

Filovirus Taxonomy

According to the International Committee on Taxonomy of Viruses, the family *Filoviridae* is one of seven families included in the order *Mononegavirales*.[4,5] The eight members of the family *Filoviridae*, referred to as filoviruses, are assigned to the three genera—*Cuevavirus*, *Ebolavirus*, and *Marburgvirus*—based on the evolutionary/phylogenetic relationship of their coding-complete genome sequences and differences in biological properties of their virions (Figure 23-1).[4–6] The members of the three genera, referred to as cuevaviruses, ebolaviruses, and marburgviruses, respectively, also differ in their geographic distribution, virion antigenicity, and overall genome organization. The International Committee on Taxonomy of Viruses *Filoviridae* Study Group recognizes one cuevavirus, Lloviu virus (LLOV); five ebolaviruses, Bundibugyo virus (BDBV), Ebola virus (EBOV), Reston virus (RESTV), Sudan virus (SUDV), and Taï Forest virus (TAFV); and two marburgviruses, Marburg virus (MARV) and Ravn virus (RAVV) (Table 23-1, Figure 23-1).[6,7] The different isolates of each filovirus are grouped into variants, which typically correspond to viruses circulating in particular human disease outbreaks.[8] For instance, several EBOV isolates were obtained during a disease outbreak in 1976 in Zaire (Democratic Republic of Congo).[9] These isolates are more closely related to each other than to several EBOV isolates obtained during a disease outbreak in Guinea in 2014. These two groups of viruses are therefore assigned different variant names (in this case, Yambuku and Makona, respectively).[10,11] The term "strain" is reserved for nonnatural, laboratory-animal-adapted or certain cDNA-derived filoviruses.[12,13]

Filovirus Disease Nomenclature

With the exception of LLOV and RESTV, all other filoviruses are associated with severe human illness. In the *International Statistical Classification of Diseases and Related Health Problems* by the World Health Organization, human disease names are standardized internationally. In its most current version, *International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10)*, two filovirus diseases are distinguished: (1) Marburg virus disease (MVD; colloquially often referred to as Marburg hemorrhagic fever), which is caused by MARV or RAVV; and (2) Ebola virus disease (EVD; colloquially often referred to as Ebola hemorrhagic fever), which is caused by BDBV, EBOV, SUDV, or TAFV (Table 23-2).[14]

Filovirus Categorization

All filoviruses are considered World Health Organization Risk Group 4 infective microorganisms. Therefore, any research involving replicative forms of the viruses must be performed in maximum containment facilities.[15] In the United States, such facilities are designated as (animal) biosafety level 4 laboratories. Given the associated high lethality with infections and the absence of licensed medical countermeasures (MCMs), filoviruses are considered high-consequence pathogens and are therefore categorized as Centers for Disease Control and Prevention (CDC) Bioterrorism Category A Agents[16] and National Institute of Allergy and Infectious Diseases Category A Pathogens.[17] Categorized as Tier 1 Select Agents, access to replicative forms of filoviruses is highly restricted by law,[16] and their export is tightly controlled.[18]

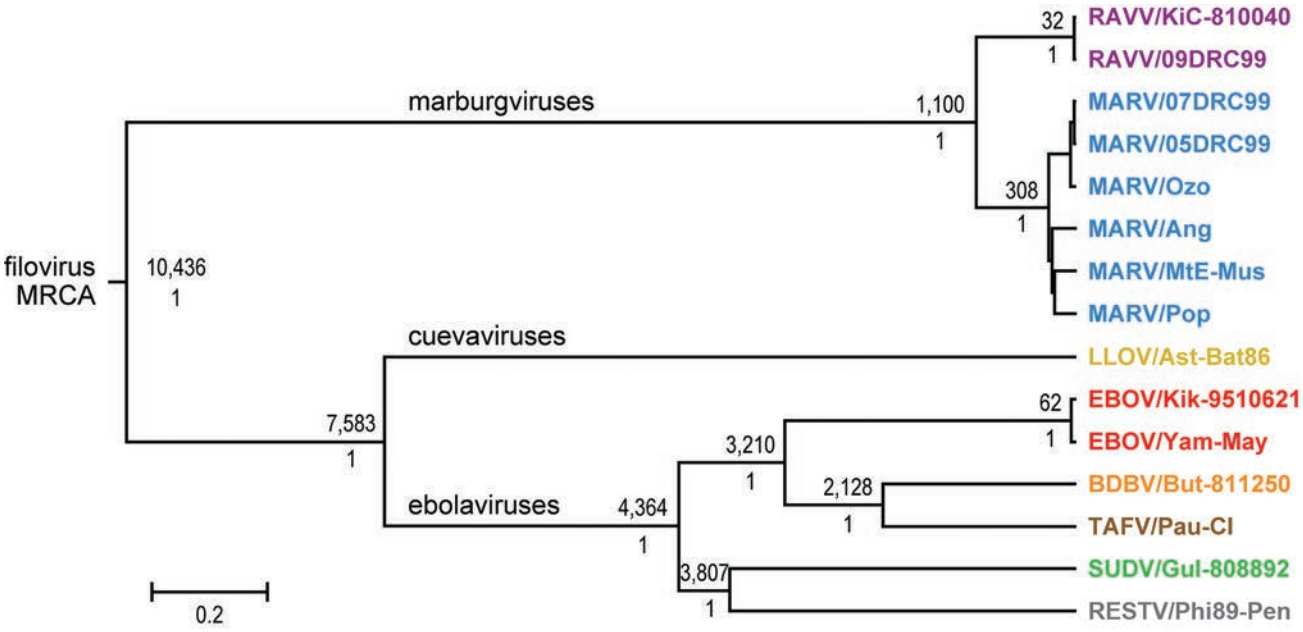


Figure 23-1. Phylogenetic relationships of members of the family *Filoviridae*. Bayesian coalescent analysis of representative cuevaviruses, marburgviruses, and ebolaviruses. Shown is the maximum clade credibility tree with the most recent common ancestor number at each node. Posterior probability values are shown beneath the most recent common ancestor estimates in years. The scale is in substitutions/site (based on data published by Serena Carroll/CDC). Appended to the virus abbreviation via a "/" is the variant abbreviation (eg, KiC, MtE, Ast, Yam, But, Pau, Gul, Phi) connected by a hyphen to the isolate designation (not all variant names are yet standardized, see data sources 3 and 4). MRCA: most recent common ancestor
Colors assigned to viruses in this table will be used in follow-up tables and figures: RAVV: *purple*; MARV: *blue*; LLOV: *yellow*; EBOV: *red*; BDBV: *orange*; TAFV: *brown*; SUDV: *green*; and RESTV: *gray*.
Data sources: (1) Carroll SA, Towner JS, Sealy TK, et al. Molecular evolution of viruses of the family *Filoviridae* based on 97 whole-genome sequences. *J Virol*. 2013;87:2608–2616. (2) Peterson AT, Bauer JT, Mills JN. Ecologic and geographic distribution of filovirus disease. *Emerg Infect Dis*. 2004;10:40–47. (3) Kuhn JH, Bao Y, Bavari S, et al. Virus nomenclature below the species level: a standardized nomenclature for natural variants of viruses assigned to the family *Filoviridae*. *Arch Virol*. 2013;158:301–311. (4) Kuhn JH, Andersen KG, Bào Y, et al. Filovirus RefSeq entries: evaluation and selection of filovirus type variants, type sequences, and names. *Viruses*. 2014;6:3663–3682.

Other countries differ from the United States and each other in the extent of implemented regulations or laws in regard to filovirus access and distribution. However, a worldwide general consensus exists on the overall threat associated with filoviruses and the need for proper containment.

MOLECULAR BIOLOGY

Filovirion Structure

Filoviruses are viruses that produce virions with filamentous morphology. Filovirions, which are enveloped particles that are greater than 800 nm long and approximately 90 nm in diameter, are covered with spike protrusions of approximately 10 nm long. The particles are flexible and appear pleomorphic, assuming shapes that are reminiscent of spaghetti (Figure 23-2), but they can also be branched or circularized.[19–25]

Filovirus Genomes and Proteins

Complete filovirions contain one or more genome copies.[23] Each genome is a monopartite, approximately 19 kb long, linear, uncapped, and polyadenylated single-stranded RNA of negative polarity that has 3′ and 5′ complementary termini. All filoviruses contain genomes with the same linear arrangement of six (LLOV) to seven genes (all other filoviruses) in the order 3′-NP-VP35-VP40-GP-VP30-VP24-L-5′.[26,27] However, the various filovirus genomes differ from each

TABLE 23-1

FILOVIRUS CLASSIFICATION AND NOMENCLATURE

2010 to Present	Outdated Virus Names and Abbreviations
Order *Mononegavirales*	
Family *Filoviridae*	
Genus *Marburgvirus*	
Species *Marburg marburgvirus*	
Virus 1: **Marburg virus (MARV)**	Marburg virus (MBGV), Lake Victoria marburgvirus
Virus 2: **Ravn virus (RAVV)**	Marburg virus (MBGV), Lake Victoria marburgvirus
Genus *Ebolavirus*	
Species *Bundibugyo ebolavirus*	
Virus: **Bundibugyo virus (BDBV)**	Bundibugyo virus (BEBOV)
Species *Reston ebolavirus*	
Virus: **Reston virus (RESTV)**	Reston ebolavirus (REBOV)
Species *Sudan ebolavirus*	
Virus: **Sudan virus (SUDV)**	Sudan ebolavirus (SEBOV)
Species *Taï Forest ebolavirus*	
Virus: **Taï Forest virus (TAFV)**	Côte d'Ivoire ebolavirus (CIEBOV), Ivory Coast ebolavirus (ICEBOV)
Species *Zaire ebolavirus*	
Virus: **Ebola virus (EBOV)**	Zaire ebolavirus (ZEBOV)
Genus *Cuevavirus*	
Species *Lloviu cuevavirus*	
Virus: **Lloviu virus (LLOV)**	

In taxonomy, taxa (orders, families, genera, and species; recognizable by italicization) are considered concepts of the mind that do not have properties. Taxa are represented by physical members, the viruses (names in color). Only virus names are to be abbreviated in technical writing. See Figure 23-1 for color explanations.
Data sources: (1) Kuhn JH, Becker S, Ebihara H, et al. Family *Filoviridae*. In: King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, eds. *Virus Taxonomy—Ninth Report of the International Committee on Taxonomy of Viruses*. London, United Kingdom: Elsevier/Academic Press; 2011:665–671. (2) Kuhn JH, Becker S, Ebihara H, et al. Proposal for a revised taxonomy of the family *Filoviridae*: classification, names of taxa and viruses, and virus abbreviations. *Arch Virol*. 2010;155:2083–2103. (3) Bukreyev AA, Chandran K, Dolnik O, et al. Discussions and decisions of the 2012–2014 International Committee on Taxonomy of Viruses (ICTV) *Filoviridae* Study Group, January 2012–June 2013. *Arch Virol*. 2014;159:821–830.

other in sequence and in the number of gene overlaps, intergenic regions, and the proteins expressed from the GP gene (Figure 23-3).[26–29] The seven filovirus genes, NP, VP35, VP40, GP, VP30, VP24, and L, encode at least seven structural proteins, respectively: nucleoprotein (NP), polymerase cofactor (VP35), matrix protein (VP40), glycoprotein ($GP_{1,2}$), transcriptional activator (VP30), secondary matrix protein (VP24), and RNA-dependent RNA polymerase (L).[23,26,27,30,31] In the case of cuevaviruses and ebolaviruses, three nonstructural proteins are encoded: secreted glycoprotein (sGP), secondary secreted glycoprotein (ssGP), and Δ-peptide (Table 23-3).[32–34] $GP_{1,2}$ is also converted into a nonstructural secreted product ($GP_{1,2\Delta}$) by tumor necrosis factor α-converting enzyme.[35] Under certain circumstances, sGP may become a structural component of ebolavirions.[36] Five of the main structural proteins—NP, VP35, VP40, $GP_{1,2}$, and L—are clearly functional analogs of the standard set of mononegaviral core proteins (N, P, M, G, and L, respectively).[37]

Filovirus Lifecycle

Filovirions bind to attachment factors on the host cell surface[38] via $GP_{1,2}$, a type 1 transmembrane and class I fusion protein,[39,40] which determines filovirus host and cell tropism.[41] After cell surface binding, filovirions enter the cell through endocytosis.[42–44] In the endolysosome, after a proteolytic cleavage that reveals the receptor-binding site, $GP_{1,2}$ engages Niemann-Pick C1 protein,[45,46] which triggers a complex $GP_{1,2}$ refolding process ensuing in fusion of the endolysosomal membrane and the virion envelope.[47] The result of this fusion is the release of the filovirus ribonucleoprotein (RNP) complex into the cytosol, where filovirus replication occurs.

At the core of the RNP complex is a helical polymer of NPs that serves as a scaffold for the filovirus genome and VP40 and VP24, which wrap around the helix.[21–23] The functional polymerase complex, which is part of the RNP, consists of filoviral L, VP35, and filovirus-unique VP30, and is bound to the filoviral genome.[48,49]

TABLE 23-2

FILOVIRUS DISEASE CLASSIFICATION AND NOMENCLATURE

ICD-10 (1990–Present)	Informal Designations
A98.3: Marburg virus disease (MVD) Caused by: **Marburg virus (MARV)** **Ravn virus (RAVV)**	Marburg hemorrhagic fever (MHF)
A98.4: Ebola virus disease (EVD) Caused by: **Bundibugyo virus (BDBV)** **Ebola virus (EBOV)** **Sudan virus (SUDV)** **Taï Forest virus (TAFV)**	Ebola hemorrhagic fever (EHF)

See Figure 23-1 for color explanations.
ICD-10: *International Statistical Classification of Diseases and Related Health Problems, Tenth Revision*
Data source: World Health Organization. *International Statistical Classification of Diseases and Related Health Problems, Tenth Revision (ICD-10)*. http://apps.who.int/classifications/icd10/browse/2015/en. Accessed September 22, 2015.

Upon release into the cytosol, these complexes move along the filoviral genome in the infected cell and transcribe the six/seven filoviral genes into polyadenylated typically monocistronic mRNAs that are then translated, or replicate the entire NP-encapsidated genome into encapsidated antigenomes and back into encapsidated progeny genomes.[50,51] VP40 and VP24, which regulate these two processes,[52] also regulate virion morphogenesis in the cell by recruiting newly formed RNPs and play major parts in the filovirion budding process from host cell membranes. Filoviruses bud through endosomal multivesicular bodies followed by exocytic release or via direct budding through the plasma membrane at membrane/lipid rafts.[53–57] $GP_{1,2}$ is expressed and proteolytically cleaved into its two subunits (GP_1 and GP_2) during transport through the secretory pathway of the infected cell, and trimers of GP_1-GP_2 heterodimers are transported to and inserted into host cell membranes.[58,59] Budding filovirions, which acquire their envelopes from the host cell membrane during egress, therefore also acquire the inserted $GP_{1,2}$, which are the spikes seen on the filovirion surface in electron microscopy sections.

Geographic Distribution

The still undefined geographic distribution of filoviruses in nature is deduced from natural host reservoir studies, epizootiology, epidemiology, serological surveys, and ecological niche modeling.[2]

Natural Reservoirs of Filoviruses

Although numerous studies were performed,[2] the natural host(s) for BDBV, EBOV, LLOV, RESTV, SUDV, and TAFV remain elusive. MARV and RAVV are the only filoviruses for which at least one natural

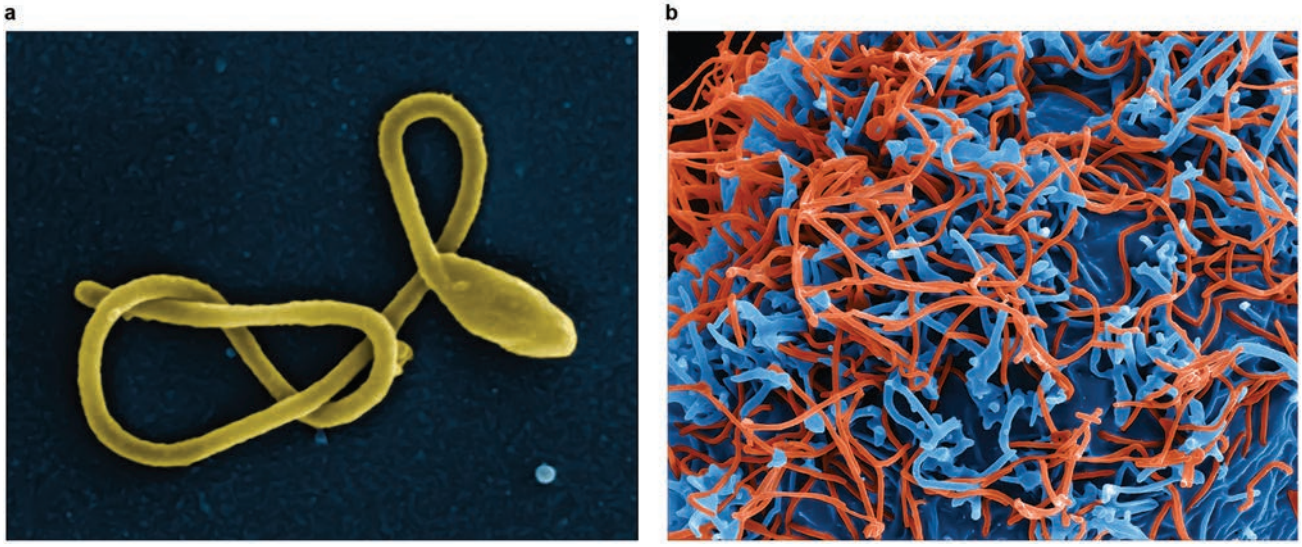


Figure 23-2. Filovirion structure. (**a**) Colorized scanning electron micrograph of a single filamentous Ebola virion (original magnification × 100,000). (**b**) Colorized scanning electron micrograph of filamentous Ebola virions (*red*) budding from a chronically infected grivet (Vero E6) cell (*blue*) (original magnification × 35,000).
Photographs: Courtesy of John Bernbaum and Jiro Wada, Integrated Research Facility at Fort Detrick, Maryland.

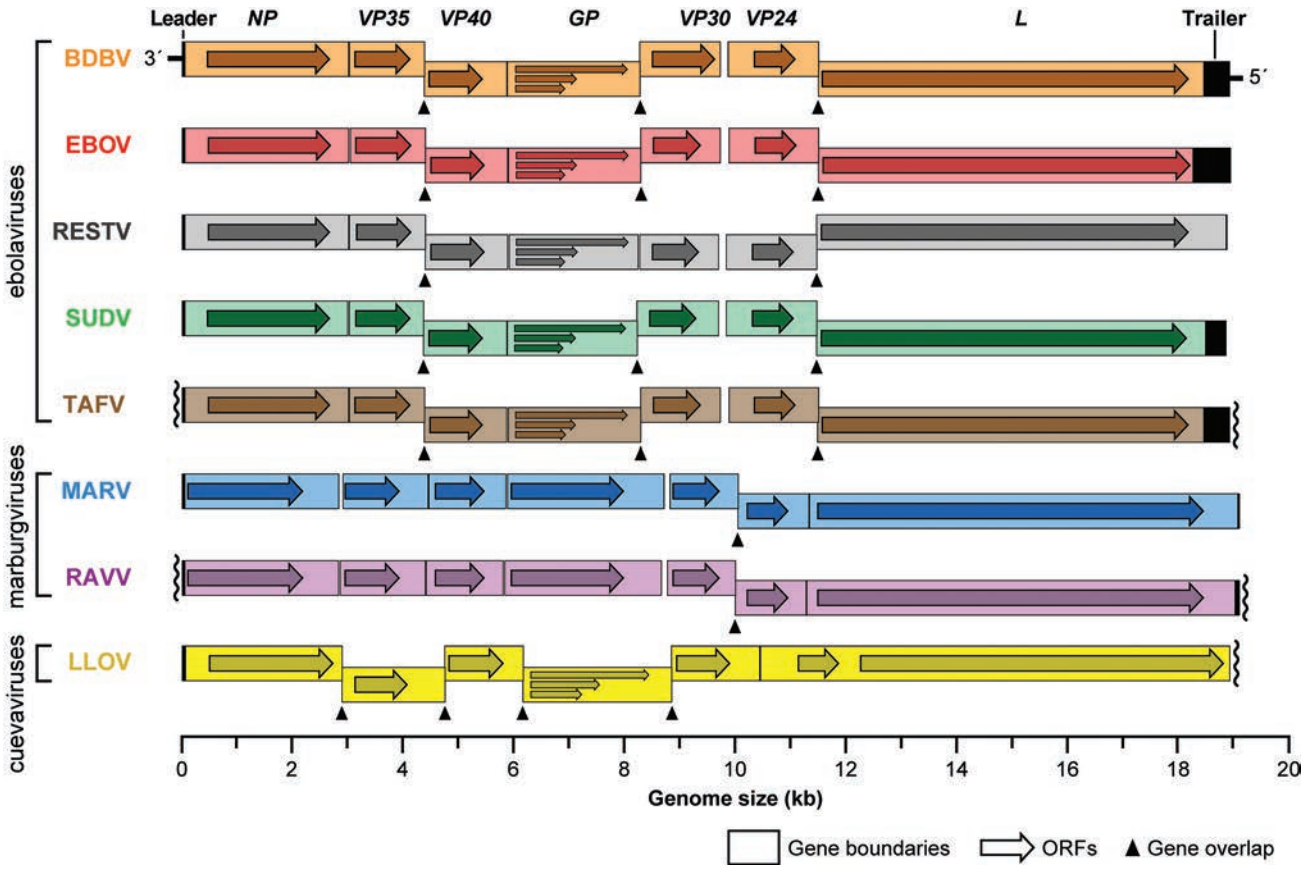


Figure 23-3. Filovirus genome organization. All filovirus genomes have the same overall sequence of genes (*rectangles*) and open reading frames (*horizontal arrows*), but differ from each other in the number and position of gene overlaps (*triangles*) and intergenic regions. Cuevaviruses and ebolaviruses differ from marburgviruses in that their GP genes contain three—rather than one—open reading frames that are accessible through transcriptional editing. Cuevaviruses differ from ebolaviruses and marburgviruses in that VP24 and L open reading frames are transcribed from a single bicistronic transcript. Genomes are drawn to scale; *waved lines* indicate incomplete sequencing of 3′ and 5′ leader and trailer sequences. See Figure 23-1 for color explanations. ORF: open reading frame

host has been unambiguously identified. Both viruses could be isolated repeatedly from several wild and seemingly healthy Egyptian rousettes (cavernicolous and frugivorous pteropodid bats of the species *Rousettus aegyptiacus*) inhabiting Kitaka Cave and Python Cave in Uganda.[60,61] A few human MVD cases were recorded among visitors of these caves.[60,61] Studies suggest that low-level transmission of both viruses among these bats occurs throughout the year with peaks of infection in older juveniles.[60] Experimental infections of Egyptian rousettes demonstrated their capacity for oral shedding of MARV,[62] suggesting that half-eaten and thereby contaminated fruit could be part of a bat–human transmission route. However, Egyptian rousettes are widely distributed across sub-Saharan and Northern Africa and Western and Southern Asia in colonies reaching up to 50,000 bats. Consequently, it is puzzling why MVD outbreaks among humans are rare events that seem to be confined to a few geographic zones of Africa.[63]

Only a loose association with bats is indicated in the cases of EBOV and RESTV. For instance, anti-EBOV immunoglobulin G antibodies or extremely short (≈300 nt) EBOV genomic fragments were detected in individual bats of several pteropodid species collected in Gabon, Ghana, and Republic of the Congo, but never both at the same time.[64–67] Anti-RESTV immunoglobulin G was detected in pteropodid bats sampled in the Philippines.[68] However, neither replicating isolates nor coding-complete genomes have yet been recovered from any bat, which is puzzling given that filoviruses generally replicate to high titers in standard cell cultures.[63] Potentially, these bats have only been exposed to, rather than infected with, EBOV/RESTV by a yet unidentified host. Finally, any connection to healthy bats is lacking for BDBV, LLOV, SUDV, and TAFV.[63]

TABLE 23-3

FUNCTION OF FILOVIRAL PROTEINS

Protein	Encoding Gene	Protein Characteristics	Protein Function
Nucleoprotein (NP)[1–8]	NP	Second-most abundant protein in infected cells and in virions; consists of two distinct functional modules; homooligomerizes to form helical polymers; binds to genomic and antigenomic RNA, VP35, VP40, VP30, and VP24; phosphorylated; depending on filovirus, *O*-glycosylated and/or sialylated	Major RNP component; nucleocapsid and cellular inclusion body formation; encapsidation of filovirus genome and antigenome; genome replication and transcription
Polymerase cofactor = viral protein 35 (VP35)[2,9–23]	VP35	Homooligomer; phosphorylated; binds to double-stranded RNA, NP, and L	RNP component; Replicase-transcriptase cofactor; inhibits innate immune response by interfering with MDA-5 and RIG-1 pathways, IRF-3 and IRF-7, and the RNAi pathway
Matrix protein = viral protein 40 (VP40)[11,12,24–26,27–42]	VP40	Most abundant protein in infected cells and in virions; consists of two distinct functional modules; homooligomerizes to form dimers and circular hexamers and octamers; binds single-stranded RNA, α-tubulin, VP35; hydrophobic; membrane-associated; contains one (marburgviruses) or three (cuevaviruses and ebolaviruses) late-budding motifs; binds NEDD4 and Tsg101; ubiquitinylated	Matrix component; regulation of genome transcription and replication; regulation of virion morphogenesis and egress; sequence determines filovirus pathogenicity in rodents. Marburgviruses only: inhibits innate immune response by JAK1 signaling
Cuevaviruses and ebolaviruses only: secreted glycoprotein (sGP)[43–46]	GP	Mostly nonstructural; secreted as a parallel homodimer in high amounts from infected cells; *N*-glycosylated, C-mannosylated, sialylated	Unknown. Hypothesized to be an antibody-decoy and antiinflammatory agent
Glycoprotein ($GP_{1,2}$)[47–60]	GP	Type 1 transmembrane and class I fusion protein; cleaved to GP1 and GP2 subunits that heterodimerize; mature protein is a trimer of $GP_{1,2}$ heterodimers; inserts into membranes; heavily *N*- and *O*-glycosylated, acylated, phosphorylated. Tumor necrosis factor α-converting enzyme (TACE) converts $GP_{1,2}$ into a soluble form ($GP_{1,2\Delta}$)	Virion adsorption to filovirus-susceptible cells via cellular attachment factors; determines filovirus cell and tissue tropism; induction of virus-cell membrane fusion subsequent to endolysosomal binding to NPC1; inhibits innate immune response by interfering with tetherin. Function of $GP_{1,2\Delta}$ is unknown
Cuevaviruses and ebolaviruses only: secondary secreted glycoprotein (ssGP)[61]	GP	Nonstructural; secreted as a glycosylated monomer	Unknown
Cuevaviruses and ebolaviruses only: Δ-peptide[62–64]	GP	Nonstructural; secreted; largely unstructured; *O*-glycosylated and sialylated	Unknown. Hypothesized to act as a suppressor of filoviral superinfection and/or as a viroporin
Transcriptional activator = viral protein 30 (VP30)[65–75]	VP30	Hexameric zinc finger protein; binds single-stranded RNA, NP, and L; phosphorylated	RNP component Cuevaviruses and ebolaviruses only: transcription initiation, reinitiation, and antitermination

(**Table 23-3** *continues*)

Table 23-3 *continued*

Secondary matrix protein = viral protein 24 (VP24)[66,68,76–86]	VP24	Homotetramerizes; hydrophobic and membrane-associated	Matrix component; regulation of genome transcription and replication; regulation of virion morphogenesis and egress; sequence determines filovirus pathogenicity in rodents Cuevaviruses and ebolaviruses only: Blocks phosphorylation of MAPK and prevents karyopherin shuttling from cytoplasm into the nucleus; inhibits host-cell signaling downstream of IFN-$\alpha/\beta/\gamma$
RNA-dependent RNA polymerase = large protein (L)[2,23,67,87–91]	L	Homodimerizes; binds to genomic and antigenomic RNA, VP35, and VP30; contains ATP-binding sites and a cap-1 MTAse domain	RNP component; genome replication and transcription; transcriptional editing

ATP: adenosine triphosphate
IFN: interferon
IRF: interferon regulatory factor
JAK1: Janus kinase 1
MAPK: mitogen-activated protein kinase
MDA-5: melanoma differentiation-associated protein-5
MTAse: methyltransferase
NEDD4: neural precursor cell-expressed, developmentally down-regulated protein 4
NPC1: Niemann-Pick C1 protein
RIG-1: retinoic acid-inducible gene-1
RNA: ribonucleic acid
RNAi: RNA interference
RNP: ribonucleoprotein complex
Tsg101: tumor susceptibility gene 101 protein
Data sources: (1) Becker S, Rinne C, Hofsäss U, Klenk HD, Mühlberger E. Interactions of Marburg virus nucleocapsid proteins. *Virology*. 1998;249:406–417. (2) Mühlberger E, Lötfering B, Klenk HD, Becker S. Three of the four nucleocapsid proteins of Marburg virus, NP, VP35, and L, are sufficient to mediate replication and transcription of Marburg virus-specific monocistronic minigenomes. *J Virol*. 1998;72:8756–8764. (3) Sanchez A, Kiley MP, Klenk HD, Feldmann H. Sequence analysis of the Marburg virus nucleoprotein gene: comparison to Ebola virus and other non-segmented negative-strand RNA viruses. *J Gen Virol*. 1992;73(Pt 2):347–357. (4) Lötfering B, Mühlberger E, Tamura T, Klenk HD, Becker S. The nucleoprotein of Marburg virus is target for multiple cellular kinases. *Virology*. 1999;255:50–62. (5) Kolesnikova L, Mühlberger E, Ryabchikova E, Becker S. Ultrastructural organization of recombinant Marburg virus nucleoprotein: comparison with Marburg virus inclusions. *J Virol*. 2000;74:3899–3904. (6) Huang Y, Xu L, Sun Y, Nabel GJ. The assembly of Ebola virus nucleocapsid requires virion-associated proteins 35 and 24 and posttranslational modification of nucleoprotein. *Mol Cell*. 2002;10:307–316. (7) Noda T, Hagiwara K, Sagara H, Kawaoka Y. Characterization of the Ebola virus nucleoprotein-RNA complex. *J Gen Virol*. 2010;91(Pt 6):1478–1483. (8) Dziubanska PJ, Derewenda U, Ellena JF, Engel DA, Derewenda ZS. The structure of the C-terminal domain of the Zaire ebolavirus nucleoprotein. *Acta Crystallogr D Biol Crystallogr*. 2014;70(Pt 9):2420–2429. (9) Cárdenas WB, Loo YM, Gale M Jr, et al. Ebola virus VP35 protein binds double-stranded RNA and inhibits alpha/beta interferon production induced by RIG-I signaling. *J Virol*. 2006;80:5168–5178. (10) Kimberlin CR, Bornholdt ZA, Li S, Woods VL Jr, MacRae IJ, Saphire EO. Ebolavirus VP35 uses a bimodal strategy to bind dsRNA for innate immune suppression. *Proc Natl Acad Sci U S A*. 2010;107:314–319. (11) Basler CF, Mikulasova A, Martinez-Sobrido L, et al. The Ebola virus VP35 protein inhibits activation of interferon regulatory factor 3. *J Virol*. 2003;77:7945–7956. (12) Bukreyev AA, Volchkov VE, Blinov VM, Netesov SV. The VP35 and VP40 proteins of filoviruses. Homology between Marburg and Ebola viruses. *FEBS Lett*. 1993;322:41–46. (13) Basler CF, Wang X, Mühlberger E, et al. The Ebola virus VP35 protein functions as a type I IFN antagonist. *Proc Natl Acad Sci U S A*. 2000;97:12289–12294. (14) Feng Z, Cerveny M, Yan Z, He B. The VP35 protein of Ebola virus inhibits the antiviral effect mediated by double-stranded RNA-dependent protein kinase PKR. *J Virol*. 2007;81:182–192. (15) Haasnoot J, de Vries W, Geutjes EJ, Prins M, de Haan P, Berkhout B. The Ebola virus VP35 protein is a suppressor of RNA silencing. *PLoS Pathog*. 2007;3:e86. (16) Johnson RF, McCarthy SE, Godlewski PJ, Harty RN. Ebola virus VP35-VP40 interaction is sufficient for packaging 3E-5E minigenome RNA into virus-like particles. *J Virol*. 2006;80:5135–5144. (17) Leung DW, Prins KC, Borek DM, et al. Structural basis for dsRNA recognition and interferon antagonism by Ebola VP35. *Nat Struct Mol Biol*. 2010;17:165–172. (18) Luthra P, Ramanan P, Mire CE, et al. Mutual antagonism between the Ebola virus VP35 protein and the RIG-I activator

(**Table 23-3** *continues*)

Table 23-3 *continued*

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(**Table 23-3** *continues*)

Table 23-3 *continued*

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Epizootiology of Filoviruses

Filoviruses are highly virulent pathogens for humans. Experimentally, most of them also cause frequently fatal infections in all thus-far studied nonhuman primates (NHPs): common marmosets (*Callithrix jacchus*), crab-eating macaques (*Macaca fascicularis*), grivets (*Chlorocebus aethiops*), hamadryas baboons (*Papio hamadryas*), rhesus monkeys (*Macaca mulatta*), and common squirrel monkeys (*Saimiri sciureus*). After serial laboratory adaptation of filoviruses, rodents such as laboratory mice, guinea pigs (*Cavia porcellus*), and Syrian golden hamsters (*Mesocricetus auratus*) infected with adapted filoviruses can develop fatal infections.[2,69,70] However, whether filoviruses are also natural pathogens for animals other than humans remains under discussion.

Five filoviruses have been loosely associated with epizootics. MARV was discovered in 1967 in West Germany among sick and dying laboratory workers who had used captive grivets imported from Uganda for poliomyelitis vaccine development.[71] However, it was never clarified at what point these monkeys became infected in captivity (Uganda or en route to West Germany) or before capture.[72] No evidence indicates that grivets are infected with filoviruses in the wild.

RESTV was discovered in 1989 when crab-eating macaques coinfected with a simian arterivirus (*Arteriviridae*: *Arterivirus*) were imported from the Philippines into the United States, fell sick, and died.[73] Similar epizootics among captive crab-eating macaques imported from the same Philippine facility occurred in the United States in 1990 and 1996, and in Italy in 1992.[74] In 2008, RESTV was identified in the Philippines in captive domestic pigs (*Sus scrofa*) coinfected with another arterivirus, porcine reproductive and respiratory disease syndrome virus. These pigs suffered and died from a respiratory and abortion disease.[75] It remains unclear how RESTV was introduced into these animal populations and why only four such introductions occurred. Although domestic pigs can be infected experimentally with EBOV[76,77] and experimentally infected piglets can transmit EBOV directly to cohoused crab-eating macaques,[78] evidence of natural infection of wild suids with filoviruses is lacking.

Indirect evidence for natural nonhuman animal filovirus infections exists for EBOV and LLOV. In the case of EBOV, catastrophic declines of central chimpanzee (*Pan troglodytes troglodytes*) and western lowland gorilla (*Gorilla gorilla gorilla*) populations correlated with EBOV-caused EVD epidemics in Gabon and in the Republic of the Congo.[79–81] In addition, duiker (*Cephalophus* spp) populations seem to have been affected at the same time. However, supporting evidence of EBOV involvement in these epizootics is limited to polymerase chain reaction (PCR)-based detection of short genomic fragments and detection of antigen in three central chimpanzees, 10 western lowland gorillas, and one duiker.[81] Replicating EBOV isolates have not yet been obtained, and complete or coding-complete EBOV genomes have yet to be detected to directly prove animal infection and possibly a link between human and animal disease.

LLOV, however, was discovered in wild animals. A coding-complete LLOV genome was assembled from tissues taken from insectivorous Schreibers' long-fingered bats (*Miniopterus schreibersii*). These bats were among hundreds that died of an unknown cause in 2002 in Cueva del Lloviu in Spain.[82] However, in the absence of a replicating LLOV isolate, determining whether LLOV caused the bat fatalities or whether the bats were infected subclinically with the virus and died of different causes is impossible.

The only direct evidence for filovirus infection of animals in the wild exists for TAFV. In 1994, a viral hemorrhagic fever-like epizootic killed most members of a wild western chimpanzee (*Pan troglodytes verus*) community in Taï National Park in Côte d'Ivoire (Western Africa).[83] A female ethologist accidentally infected herself with the viral-hemorrhagic-fever-causing pathogen while performing necropsies on the deceased animals. TAFV was isolated from clinical material, and serological testing demonstrated that western chimpanzees were infected with the same agent.[84] It is unclear, however, how the chimpanzees became infected and whether such infections are common or unusual events.

Epidemiology of Filovirus Infections

Filoviruses were discovered in 1967 in West Germany.[71] Since then, 37 human EVD and MVD outbreaks have been recorded (Figure 23-4).[85] The incidence of MVD and EVD apparently has continued to increase over the years, but this increase may simply result from improved surveillance and reporting. Statistical support that any ebolavirus is more virulent than another is scant, although based on current case numbers, MVD appears to be more lethal compared to EVD (Figure 23-4). Close to all of the 37 filovirus disease outbreaks occurred in Middle/Eastern Africa. Interestingly, "hot spots" for filovirus disease outbreaks seem to exist. For instance, EBOV reappears continuously in Gabon, Republic of the Congo, and western Democratic Republic of the Congo; whereas BDBV, SUDV, and MARV caused repeated outbreaks in the northeastern Democratic Republic of the Congo, southern South Sudan, and Uganda (Figure 23-5).

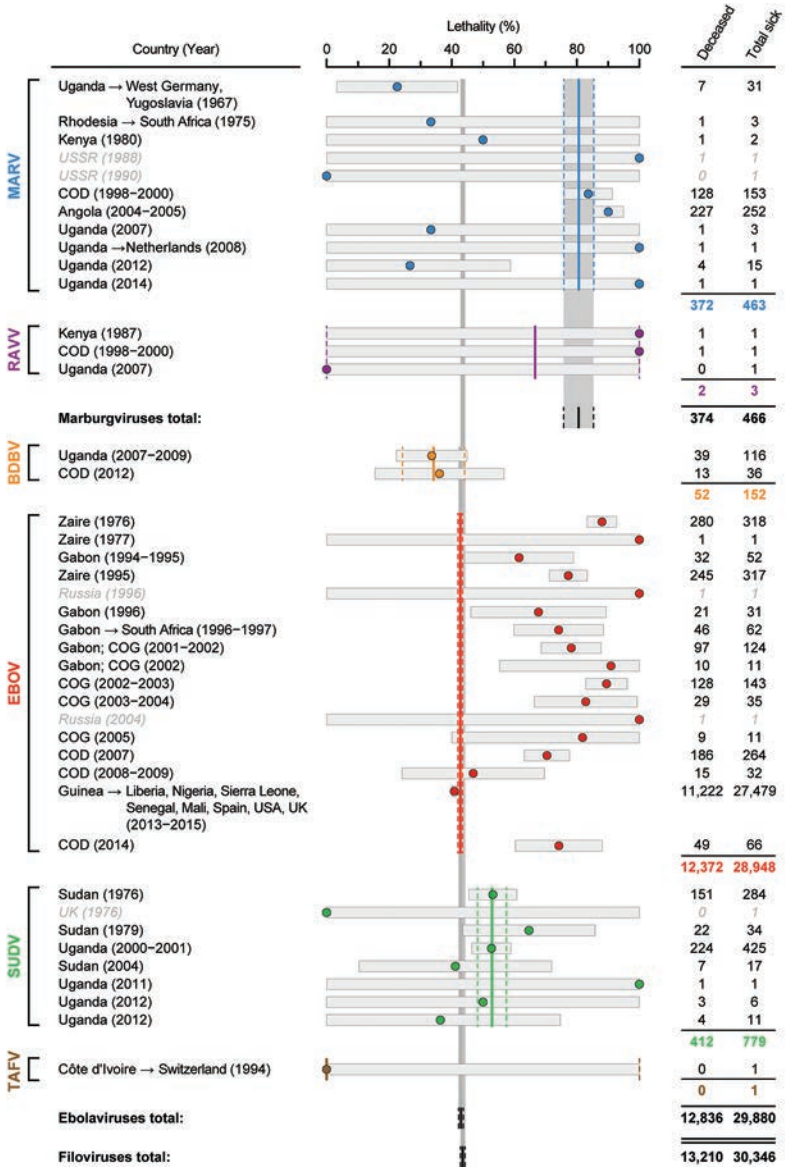


Figure 23-4. Ebola and Marburg virus disease outbreaks. Ebola virus disease and Marburg virus disease outbreaks are listed chronologically by virus (*colored vertically on the left*). International case exportations are pointed out by *arrows*; proven laboratory infections are highlighted in *gray and italics*. Total case numbers and total number of fatalities are itemized for each outbreak in the utmost right columns (updated from data sources 1 and 2). The lethality/case fatality rate (*dots*) for each outbreak is plotted in the middle column on a 0% to 100% scale along with 99% confidence intervals (*gray horizontal bars*). The average lethality of a particular virus or virus group is shown by *vertical lines* (99% confidence intervals are emphasized by *dashed lines*). The vertical line showing the average lethality of all Ebola virus disease outbreaks overlaps with the vertical line showing the average lethality of all filovirus disease outbreaks and the vertical line showing the average lethality of all disease outbreaks caused only by Ebola virus (*red*). At the time of this writing, the 2013–2015 Ebola virus-caused Ebola virus disease outbreak in Western Africa has not been brought under control. Consequently, the case and fatality numbers are still subject to change and lethality should rather be regarded as a proportion of fatal cases than lethality until final numbers become available. See Figure 23-1 for color explanations. COD: Democratic Republic of the Congo; COG: Republic of the Congo; UK: United Kingdom; USSR: Union of Soviet Socialist Republics.

Data sources: (1) Kuhn JH. *Filoviruses: A Compendium of 40 Years of Epidemiological, Clinical, and Laboratory Studies*. In: Calisher CH, ed. *Archives of Virology* Supplementa Series, Vol 20. Vienna, Austria: Springer-Verlag Wien; 2008. (2) Kuhn JH, Dodd LE, Wahl-Jensen V, Radoshitzky SR, Bavari S, Jahrling PB. Evaluation of perceived threat differences posed by filovirus variants. *Biosecur Bioterror*. 2011;9:361–371. (2) Kuhn JH. Ebolavirus and Marburgvirus infections. In: Kasper DL, Fauci AS, Hauser SL, Longo DL, Jameson JL, Loscalzo J, eds. *Harrison's Principles of Internal Medicine*. Vol 2. 19th ed. Columbus, OH: McGraw-Hill Education; 2015:1323–1329.

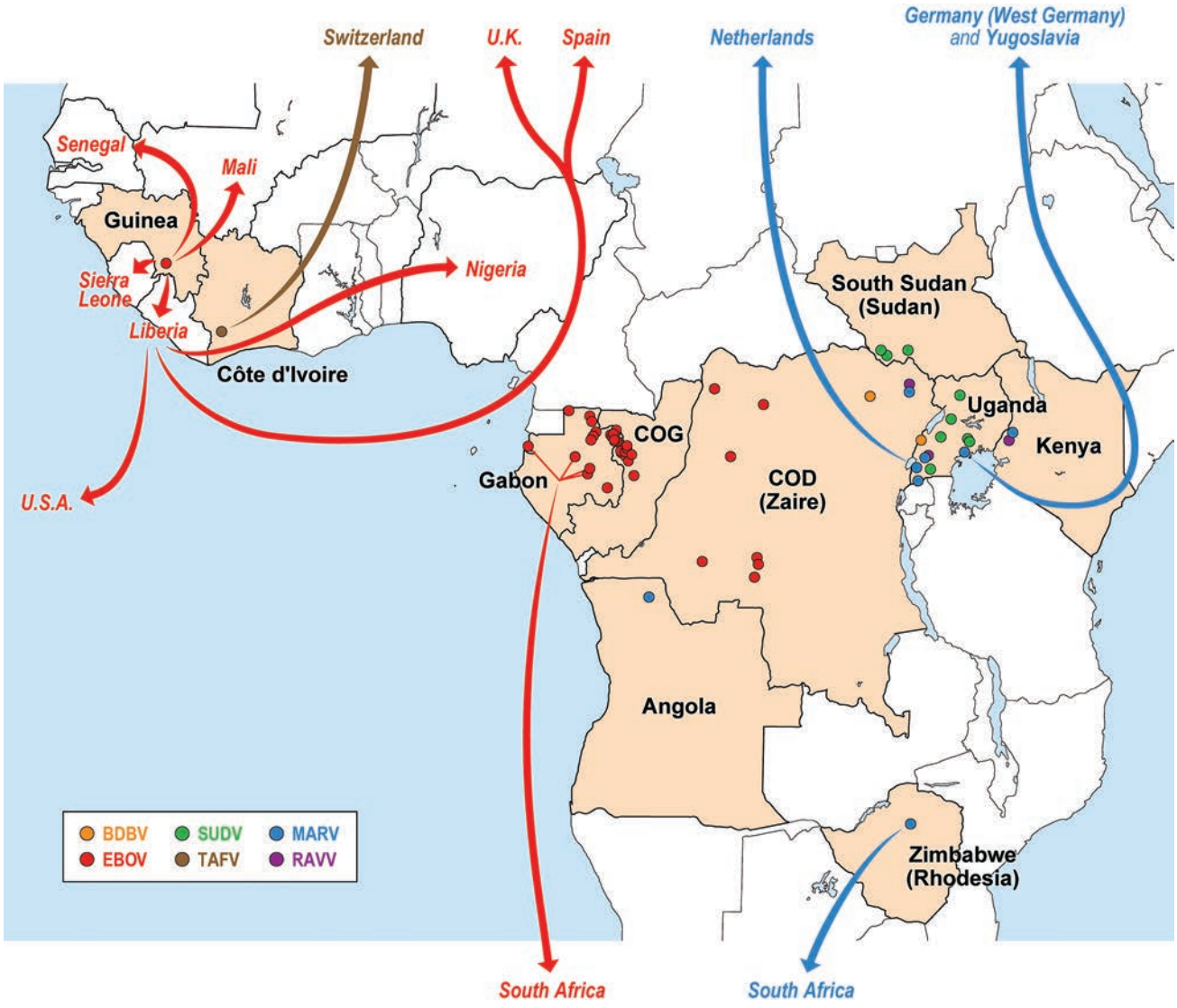


Figure 23-5. Ebola and Marburg virus disease outbreaks. Middle/equatorial African countries affected by Ebola virus disease and/or Marburg virus disease outbreaks are shown in *light brown* with outbreak locations marked as dots colored according to the etiological filovirus (updated from data sources 1 and 2). *Arrows* mark international case exportation. Former country names are listed in parentheses under the present name.
COD: Democratic Republic of the Congo; COG: Republic of the Congo.
Data sources: (1) Kuhn JH. *Filoviruses: A Compendium of 40 Years of Epidemiological, Clinical, and Laboratory Studies*. In: Calisher CH, ed. *Archives of Virology* Supplementa Series, Vol 20. Vienna, Austria: Springer-Verlag Wien; 2008. (2) Kuhn JH, Dodd LE, Wahl-Jensen V, Radoshitzky SR, Bavari S, Jahrling PB. Evaluation of perceived threat differences posed by filovirus variants. *Biosecur Bioterror*. 2011;9:361–371. (3) Kuhn JH. Ebolavirus and Marburgvirus infections. In: Kasper DL, Fauci AS, Hauser SL, Longo DL, Jameson JL, Loscalzo J, eds. *Harrison's Principles of Internal Medicine*. Vol 2. 19th ed. Columbus, OH: McGraw-Hill Education; 2015:1323–1329.

Almost all filovirus disease outbreaks began with a single introduction of a filovirus into an index case who subsequently transmitted the infection to other humans. Thus, initial human filovirus infections are extremely rare events and occurred probably less than 50 times since 1967.[2] In general, past filovirus disease outbreaks occurred in rural and often secluded areas and affected only several dozens to a few hundred people.[2] However, a few outbreaks occurred in populated areas, such as the 1995 EVD outbreak caused by EBOV in Kikwit (Zaire) and the 1998–2000 MVD outbreaks from MARV and RAVV around Durba and Watsa, Democratic Republic of the Congo (the former Zaire). This pattern shifted dramatically in December

2013 when the largest EVD outbreak began in Western Africa from a single introduction of EBOV.[86,87] As of October 11, 2015, this outbreak has thus far caused 28,490 cases and 11,312 deaths in Guinea, Liberia, Mali, Nigeria, Senegal, and Sierra Leone (Figure 23-4).

Serological Surveys

Numerous serological surveys for antibodies against filoviral antigens have been performed in human and animal populations to further define the geographic spread of filoviruses and to better estimate risk of infection.[2] However, results of most of these surveys are puzzling. In some surveys, the seroprevalence of anti-EBOV antibodies is extremely high (>5%–20%) in humans indicating frequent exposure to EBOV or related agents in the absence of disease. In other surveys, the seroprevalence of antifilovirus antibodies is moderate among humans living in areas that never had filovirus disease outbreaks (eg, certain African countries, Belarus, Germany, Ukraine). Many of these studies used MARV, EBOV, or SUDV antigens in indirect immunofluorescence assays (IFAs), which are subjective and thus difficult to interpret. IFA serosurveys are therefore regarded as presumptive by most experts. Modern serosurveys rely on the use of enzyme-linked immunosorbent assays (ELISAs) in conjunction with confirmatory western blot for the detection of antifilovirus antibodies. Few such studies were published, and results of these studies most often did not confirm IFA results.[2]

Overall, three disparate possibilities arise from the performed serosurveys (IFA and/or ELISA). First, all obtained results may be artifacts based on common nonfilovirus antibodies in human sera that are cross-reactive with the used filoviral antigens, thereby leading to false-positive results. Second, filoviruses could subclinically infect humans or cause only mild disease, thereby leading to high seropositivity rates. Current data on the possibility of such infections are scarce[88,89] and hotly debated, but the currently ongoing EVD outbreak in Western Africa may reveal subclinical infections resulting from the sheer number of recorded infections. Third, the discovery of LLOV in Spain[82] indicates the possibility that filovirus diversity and geographic distribution is broader than currently appreciated. Perhaps contact with possibly nonpathogenic filoviruses (eg, LLOV- or RESTV-related viruses) induces antibodies that are cross-reactive with closely related filoviral antigens. Without convincing data for any of these possibilities, serosurvey data should not be ignored, but they should be used with caution for prediction of filovirus distribution or infection risk assessments.

Environmental Niche Modeling

Environmental niche modeling (ie, the use of algorithms to predict the geographic distribution of organisms on the basis of their environmental distribution using meteorological and other data) indicates succinct distributions for filoviruses in the Afrotropic ecozone.[90–95] According to these models, ebolaviruses are endemic in humid rain forests in Western and Middle Africa and South-Eastern Asia, whereas marburgviruses circulate in caves located in arid woodlands in Middle, Eastern, and Southern Africa.[90,91] Filovirus emergence in human populations appears to be associated with the appearance of climate anomalies or drastic climate changes.[92] For instance, ebolavirus activity is suggested to be correlated with unusually heavy rainfalls subsequent to extended dry periods.[90,94,95] If these models prove correct, then filovirus disease outbreaks should be expected in numerous African countries that have thus far not experienced (or noticed) any outbreaks.[93]

TRANSMISSION

As the natural reservoir hosts for most filoviruses are unknown, how filoviruses are introduced into the human population is unclear. Researchers are tempted to speculate that initial infections occur after direct contact with tissues, secretions, or excretions of an animal or after a bite or sting.[96,97] Even in the case of human infections in Ugandan caves that harbored MARV- and RAVV-infested Egyptian rousettes,[60,61] it remains to be explained how these few people became infected, and why many others who visited these caves did not.

Human-to-human spread of filovirus infections is better understood. Epidemiological studies clearly demonstrate that filovirus transmission almost exclusively occurs through direct person-to-person contact or through direct contact with filovirus-contaminated material.[2,98,99] Airborne spread has not been demonstrated for any filovirus during a natural outbreak, although healthcare workers risk infection during artificial aerosol creation performed as part of medical procedures such as centrifugation of samples, intubation of patients, or suction used during surgical procedures.[100,101]

Filoviruses replicate in humans to high titers (>10^6 plaque-forming units/mL) and at least in the case of EBOV, vast quantities of antigen deposit in the skin and around skin appendages.[102] In animal models, the LD_{50} of EBOV has been estimated to be as low as 1 plaque-forming unit.[103] As a consequence, filoviruses

are highly infectious and readily contagious through close contact with skin or mucous membranes, especially in the presence of small lesions.[104–107] Filoviruses or filovirus RNA may be present in genital, nasal, and other bodily secretions. Transmission appears to be a rare event during the early, asymptomatic phase of disease.[100,101] However, in the absence of personal protective equipment (PPE; disposable gowns, gloves, shoe covers, face-shields, or goggles), transmission occurs readily. Transmission is typical between sick people and their family members, friends, or healthcare workers who care for them; between deceased people and people who prepare bodies for funerals; and between medical personnel who handle medical samples, contaminated medical equipment, or decontamination. A second important transmission pathway is nosocomial spread through contaminated and reused disposable needles and syringes that, unfortunately, is still common in many chronically underfunded and therefore underequipped African hospitals.[100,101,104,108–111] Implementation of quarantine measures and use of proper PPE usually suffice to interrupt human-to-human transmission and to terminate outbreaks.[100,101,105,109,110,112]

At this time, the question remains whether filoviruses truly adapt to the human host during prolonged interhuman transmission, and whether such adaptation could result in the natural selection of variants that is either more or less transmissible or more or less virulent. A recent study performed during the 2013–2015 EVD outbreak in Africa indicates that mutations accumulate and particular subpopulations of EBOV arise during transmission,[87] but these subpopulations have not been associated with particular phenotypes.

THREAT TO THE WARFIGHTER

The warfighter could potentially be at risk of filovirus infection during humanitarian deployment, military campaigns, or war. Exposure to filoviruses could occur coincidentally through contact with unknown filovirus reservoir(s) and accidentally through exposure to infected people, deceased patients, or materials contaminated with human secretions or excretions. In addition, the warfighter may be exposed deliberately during an attack with biological weapons deployed by terrorists, hostile groups, or nation states.[113] Coincidental and accidental risks can be dramatically reduced for the warfighter if common sense practices for tourists and standard operating procedures for healthcare workers are implemented.

Filoviruses and the Soviet Biological Warfare Program

The Soviet Union maintained a highly clandestine biological weapons program from at least 1918 until at least 1991.[114,115] In 1999, a published account from a high-ranking defector of the civilian "Biopreparat" arm of this program revealed that two filoviruses, EBOV and MARV, were included in the program.[115] Additional revelations about the biological weapons program are scarce. Consequently, knowledge of the scope, goals, and achievements of especially the second generation of the program (1972–1991) is deduced from accounts from several additional defectors and a few researchers who were previously involved and legally left the Soviet Union/Russia, as well as from a few leaked classified reports or memos.[114]

Classified filovirus research probably began in the Soviet Union shortly after the discovery of MARV in 1967 in Marburg and Frankfurt, West Germany, and Belgrade, Yugoslavia. West German and Yugoslavian scientists provided several isolates of the novel virus (most notably MARV Popp) to numerous international institutes for characterization studies to counter allegations that West Germany had developed a biological weapon. Among these institutes was the Union of Soviet Socialist Republics Academy of Medical Sciences Scientific Research Institute of Poliomyelitis and Viral Encephalitides (now named the M.P. Chumakov Institute of Poliomyelitis and Viral Encephalitides of the Russian Academy of Medical Sciences) in Moscow.[114,116] Unclassified, nonmilitary-related research began at the institute immediately and resulted in a few published reports in Russian from 1968 to 1972.[2] Current thinking is that filovirus research became classified thereafter and soon was abandoned at that institute after MARV cultures were transferred to the main military virology institute, the Scientific Research Institute of Sanitation of the Union of Soviet Socialist Republics Ministry of Defense in Zagorsk (now named the Virology Center under the Scientific-Research Institute of Microbiology in the renamed city of Sergiev Posad) close to Moscow.[114]

The Institute of Tropical Medicine in Antwerp, Belgium, which had received MARV during the 1967 MVD outbreak and EBOV during the 1976 EVD outbreaks, provided MARV isolate Voege and an EBOV Yambuku isolate in the mid-1980s within a standard collaboration for diagnostics development to the Belorussian Scientific-Research Institute for Epidemiology and Microbiology of the Belorussian SSR Ministry of Health (now named the Republican Research and Practical Center for Epidemiology and Microbiology) in Minsk. Although the institute in Minsk continued international collaboration and published manuscripts

using MARV and EBOV, both viruses were most likely also transported to the institute in Zagorsk.

Probably from Zagorsk, MARV Popp and the EBOV Yambuku isolates were transferred to the highly secretive Scientific-Production Association "Vector" (now named the State Scientific Center for Virology and Biotechnology "Vector") in the closed settlement of Kolcovo close to Novosibirsk. MARV Voege and the same EBOV isolate were also transferred to the Scientific-Research Anti-Plague Institute for Siberia and the Far East in Irkutsk (Figure 23-6).[114] Judging from Russian publications released in the mid-1990s, the three institutes in Irkutsk, Kolcovo, and Zagorsk made significant progress in basic research in terms of EBOV



Figure 23-6. Locations of clandestine filovirus research in the Soviet Union. Marburg virus was provided to the Union of Soviet Socialist Republics Academy of Medical Sciences Scientific Research Institute of Poliomyelitis and Viral Encephalitides in Moscow by West German and Yugoslavian scientists and then transported to the Scientific-Research Institute of Sanitation of the Union of Soviet Socialist Republics Ministry of Defense in Zagorsk. Belgian scientists provided Marburg virus and Ebola virus to the Belorussian Scientific-Research Institute for Epidemiology and Microbiology of the Belorussian SSR Ministry of Health, which also forwarded cultures to Zagorsk. From there, cultures were transferred to the Scientific-Production Association "Vector" in Kolcovo and the Scientific-Research Anti-Plague Institute for Siberia and the Far East in Irkutsk. Major offensive research and development most likely occurred in Zagorsk and Kolcovo at least until 1991.
Data sources: (1) Zilinskas RA, Leitenberg M, with Kuhn J. *The Soviet Biological Weapons Program: A History*. Cambridge, MA: Harvard University Press; 2012. (2) Alibek K, Handelman S. *Biohazard—The Chilling True Story of the Largest Covert Biological Weapons Program in the World—Told from Inside by the Man Who Ran It*. New York, NY: Random House; 1999. (3) Zilinskas RA. The anti-plague system and the Soviet biological warfare program. *Crit Rev Microbiol*. 2006;32:47–64.

and MARV genomic sequencing, established ELISA- and PCR-based diagnostics, and developed parenteral and aerosol rodent and NHP MVD/EVD models.[114]

Little is known about bona fide offensive research and development efforts. Based on current data, EBOV research apparently did not progress beyond the research stage because of production problems.[114] MARV, however, was specified in the Soviet 11th five-year plan (1981–1985) to be weaponized.[114] Weaponization efforts probably began in 1983 at the Scientific-Production Association or "Vector," focusing on characterization of the pathogen, high-titer production in rodents and later in tissue bioreactors, and production of dried and milled formulations. Finally, these formulations were tested in aerosol experiments using animals around 1991. However, most likely few or none of the formulations reached the validation stage, and type-classified weapons (ie, MARV-loaded weapons that had been tested and succeeded in open-air testing) were not developed.[114]

The extensive effort within the Soviet biological weapons program and the ultimate failure to produce a reliable weapon indicate that the risk of attack with a biological weapon constructed to spread filoviruses is relatively low, but not negligible. Although filoviruses are not naturally airborne and transmission from person-to-person is negligible without direct contact, the Soviet program suggests that these hurdles are thought by some not to be unsurmountable. However, a large-scale attack on civilians or armed forces with filoviruses seems unlikely and possible only by nation states rather than by small adversary groups. Such groups could—in theory—attempt to introduce filoviruses into human populations by means other than weaponry (eg, direct injection with needles; self-infection) to induce panic and thereby affect the economy of target populations.[113,117] Therefore, educating the general public about filoviruses is vital to reduce the psychological impact of such an attack.

PREVENTION

Behavioral Modification

Prevention of initial introduction of filoviruses into human or other animal populations is difficult to impossible as long as the ecology of the viruses is not understood and their natural host reservoirs remain unknown. However, general "good infection control behaviors" should be encouraged to minimize the risk of initial infection. Such control behaviors include the avoidance of direct contact with wild animals; consumption of uncooked or undercooked wildlife; and unprotected exposure to animal excretions, secretions, fluids, or tissues. Control behaviors further include consuming water that has been boiled, reducing contact with arthropods (eg, application of insect repellents, using mosquito nets, screening for ticks), avoiding contact with obviously sick people, and avoiding unprotected sex. During a filovirus disease outbreak, locals should be educated about the nature of filoviruses. Certain cultural practices, such as handshaking, or particular funeral rituals, such as ritual hand washing or embalming of bodies, should either be strongly discouraged or modified to decrease filovirus transmission risk.[118–121] Quarantine of infected people and avoidance of direct person-to-person contact generally suffices to prevent further spread. Healthcare and other staff should don proper PPE before handling patients or suspected cases of filovirus infection, clinical samples, or potentially contaminated material. Strict implementation of barrier nursing techniques in patient care is also vital. N-95/N-100 and positive air pressure respirators, if available, should be used especially during clinical procedures that may generate aerosols. However, users should be aware that positive air pressure respirators may induce fear, especially among local populations. As fomites are an important route of filovirus transmission, reuse of medical equipment should be avoided whenever possible. At all times, disposables should be used only once and promptly discarded.[100,112–127]

Filovirus Inactivation and Decontamination

Filoviruses produce enveloped virions that contain single-stranded RNA genomes.[26,27] These virions, which are relatively labile, are rapidly inactivated by heat, pressure, radiation, or contact with detergents. Cheap and commonly available detergents (diethyl ether, phenolic compounds, sodium deoxycholate) and oxidizing agents, such as bleach or bleaching powder, should be used to disinfect surfaces or patient excreta or secreta.[128–132] However, despite their overall lability, filovirions are stable for several days in liquids such as drying blood and on surfaces typically found in treatment units for more than 5 days.[133] Corpses, which may contain infectious filoviruses for extended periods of time,[134] should be buried quickly. Ideally, potentially contaminated disposables are autoclaved, irradiated, or burned.

Vaccines

Despite numerous and diverse efforts,[2,135–138] no FDA-approved vaccine exists to prevent filovirus infections. Candidate vaccines include inactivated and attenuated filoviruses, subunit vaccines (adenovirus,[139–145] alphavirus,[146–150] lyssavirus,[151,152] orthopoxvirus,[153] paramyxovirus,[154–156] and vesiculovirus[157–162] vectors expressing filovirus NP, VP35, VP40, $GP_{1,2}$, VP24, VP30, and/or VP24), naked DNA vaccines encoding filovirus proteins (alone or in combination with adenovirus-based vectors),[140,163–166] and filovirus-like particles consisting only of VP40, NP, and $GP_{1,2}$.[167–170] These candidate vaccines were variably efficacious in different animal models. All of these vaccines have advantages and disadvantages in regard to safety profiles, induction of long-term immune responses, or ease of production.[2,135–138] In recent years, consensus has been reached that only platforms that are highly protective in NHP models of filovirus disease should be considered for further development.[69] Among these platforms, the most promising candidate vaccines are those that have been built using adenoviral or vesiculoviral backbones or filovirus-like particles (Table 23-4).

DISEASE

EVD and MVD are largely characterized through clinical observation of patients in underequipped hospitals,[171–178] individual observations of patients who were transported to developed countries or suffered from accidental infections,[179–183] limited examination of tissues obtained during human outbreaks via biopsies,[102,112,184] and a very low number of often incomplete autopsies.[185–192] Most of the examinations, including biopsies and autopsies, were performed before techniques for characterization of molecular pathogenesis events were available. Consequently, a paucity of EVD and MVD biomarkers exists.[193,194] Given the rarity of EVD and MVD outbreaks, disease characterization has therefore depended on the use of filovirus-susceptible animals (rodents and NHPs). Although frequently referred to as "models" of EVD and MVD, the human disease remains largely uncharacterized, and animal infections do not necessarily mimic EVD and MVD completely.[195] For instance, hemorrhagic manifestations, disseminated intravascular coagulopathy, bystander apoptosis, and lethality differ among the types of animals used as well as humans.

Wild type filoviruses do not cause disease in rodents and therefore require serial adaptation to produce disease in these rodents.[103,196] Such adaptation has been challenging, especially with laboratory mice. Consequently, true mouse models are only available for three filoviruses (ie, EBOV, MARV, RAVV).[103,197–199] Laboratory mice are frequently used for initial MCM evaluation efforts for many reasons including the following:

- Ethical concerns are limited about such experiments.
- Mice are easily maintained.
- Experiments are possible involving large numbers of animals.
- The clonal background of laboratory mice simplifies statistical analysis of observed MCM effects on infection.[200]

Golden hamsters are becoming increasingly popular as models for EBOV-induced EVD because they—in contrast to laboratory mice—develop pronounced coagulatory defects mimicking those seen in humans.[201] Guinea pigs are typically used as a bridge between small rodent (laboratory mice, hamsters) and NHP models for infections caused by EBOV and MARV.[196,202–204] In contrast to laboratory mice, MCM evaluation results recorded during guinea pig experiments often translate to similar observations in NHPs. In addition, these experiments are less expensive and not as logistically challenging as NHP experiments.

Nevertheless, NHPs are considered the gold standard for MCM evaluation, which is largely a result of requirements specified in the US Food and Drug Administration's "Animal Rule" for licensure of candidate vaccines and therapeutics that cannot be tested in human clinical trials.[69] EBOV and MARV rapidly infect crab-eating macaques, grivets, hamadryas baboons, rhesus monkeys, and common marmosets and induce a usually uniformly lethal disease.[2,69,205–210] SUDV and RAVV infections can also be studied in crab-eating macaques and rhesus monkeys,[207] whereas experiments using other NHPs have not been reported. Truly useful NHP models for BDBV and TAFV infections have yet to be described.

Pathogenesis

$GP_{1,2}$ embedded in the envelope of filovirions determines cell and therefore tissue tropism of filoviruses based on its interaction with cell surface attachment factors and the intracellular receptor, NPC1.[40] Filoviruses have a broad tropism, that is, the cognate binding partners of $GP_{1,2}$ are expressed on a wide variety of cell types[41] (in vivo, notable exceptions are lymphocytes, myocytes, and neurons[20]). In addition, cuevaviruses and ebolaviruses, but not marburgviruses, produce

TABLE 23-4

SELECTED PROMISING CANDIDATE VACCINES FOR FILOVIRUS INFECTIONS

Candidate Vaccine	Antigen	Efficacy in Nonhuman Primates	Additional Information
Filovirus-like virions (VLPs)[1]	**EBOV**-like virion consisting of **EBOV** NP, VP40, and $GP_{1,2}$	100% survival of crab-eating macaques exposed to **EBOV**	Nonreplicating protein-based vaccine; low safety risks; clinical-grade materials will be required for further development
HPIV-3 vector[2]	**EBOV** $GP_{1,2}$	100% survival of rhesus monkeys exposed to **EBOV**	Replicating, therefore safety concerns; possibly background immunity to vector
Naked DNA + recombinant adenovirus 5 (rAD5) vector[3]	**EBOV** $GP_{1,2}$ + **SUDV** $GP_{1,2}$ (in individual vectors)	Cross protection; 100% survival of crab-eating macaques exposed to **BDBV**	Nonreplicating; possibly background immunity to vector; high dose necessary
Naked DNA + recombinant adenovirus 5 (rAD5) vector[4]	**EBOV** $GP_{1,2}$	100% survival of crab-eating macaques exposed to **EBOV**	Nonreplicating; possibly background immunity to vector; high dose necessary
RABV vector[5]	**EBOV** $GP_{1,2}$	100% survival of rhesus monkeys exposed to **EBOV**	Replicating, therefore safety may be of concern
RABV vector[5]	**EBOV** $GP_{1,2}$	50% survival of rhesus monkeys exposed to **EBOV**	Nonreplicating
Recombinant chimpanzee adenovirus 3 vector (cAD3)[6]	**EBOV** $GP_{1,2}$	100% survival of crab-eating macaques exposed to **EBOV**	Nonreplicating; high dose necessary. Phase 1 clinical trials finished
Recombinant human adenovirus 5 vector (CAdVax)[7]	**EBOV** NP+$GP_{1,2}$ + **MARV** NP+ $GP_{1,2}$ + **RAVV** $GP_{1,2}$ + **SUDV** $GP_{1,2}$ (in individual vectors)	100% survival of crab-eating macaques exposed to **EBOV** or **MARV**	Nonreplicating; possibly background immunity to vector; high dose necessary. **EBOV**-exposed survivors also survived later **SUDV** exposure
Recombinant human adenovirus 5 vector (rAD5)[8,9]	**EBOV** $GP_{1,2}$	100% survival of crab-eating macaques exposed to **EBOV**	Nonreplicating; possibly background immunity to vector; high dose necessary. Phase 1 clinical trials finished
VEEV vector[10]	**EBOV** $GP_{1,2}$ + **SUDV** $GP_{1,2}$ (in individual vectors)	100% survival of crab-eating macaques exposed to **EBOV** or **SUDV**	Nonreplicating; possibly background immunity to vector; clinical-grade materials will be required for further development.
VEEV vector[10]	**SUDV** $GP_{1,2}$	100% survival of crab-eating macaques exposed to **SUDV**	Nonreplicating; possibly background immunity to vector; high dose necessary
Vesicular stomatitis Indiana virus (VSIVΔG) vector[11,12]	**EBOV** $GP_{1,2}$	100% survival of crab-eating macaques exposed to **EBOV**	Replicating, therefore safety may be of concern
Vesicular stomatitis Indiana virus (VSIVΔG) vector[11–14]	**MARV** $GP_{1,2}$	100% survival of crab-eating macaques exposed to **MARV**	Replicating, therefore safety may be of concern

(**Table 23-4** *continues*)

Table 23-4 *continued*

Vesicular stomatitis Indiana virus (VSIVΔG) vector[15]	**BDBV** $GP_{1,2}$	100% survival of crab-eating macaques exposed to **BDBV**	Replicating, therefore safety may be of concern
Vesicular stomatitis Indiana virus (VSIVΔG) vector[16]	**EBOV** $GP_{1,2}$	75% survival of crab-eating macaques exposed to **BDBV**	Replicating, therefore safety may be of concern
Vesicular stomatitis Indiana virus (VSIVΔG) vector[13]	**MARV** $GP_{1,2}$	Cross protection; 100% survival of crab-eating macaques exposed to **RAVV**	Replicating, therefore safety may be of concern
Vesicular stomatitis Indiana virus (VSIVΔG) vector[17]	**EBOV** $GP_{1,2}$ + **MARV** $GP_{1,2}$ + **SUDV** $GP_{1,2}$ (in individual vectors)	100% survival of crab-eating macaques exposed to **EBOV**, **MARV**, **SUDV**, or **TAFV**	Replicating, therefore safety may be of concern
Vesicular stomatitis Indiana virus (VSIVΔG) -vector[17]	**EBOV** $GP_{1,2}$ + **MARV** $GP_{1,2}$ + **SUDV** $GP_{1,2}$ (in individual vectors)	100% survival of rhesus monkeys exposed to **SUDV**	Replicating, therefore safety may be of concern

BDBV: Bundibugyo virus
EBOV: Ebola virus
HPIV3: human parainfluenza virus 3
MARV: Marburg virus
RABV: rabies virus
RAVV: Ravn virus
SUDV: Sudan virus
TAFV: Taï Forest virus
VEEV: Venezuelan equine encephalitis virus
Note: Information on the status of all ongoing filovirus-relevant clinical trials can be found at https://ClinicalTrials.gov with the search terms "Ebola" or "Marburg." See Figure 23-1 for color explanations.
Data sources: (1) Warfield KL, Swenson DL, Olinger GG, Kalina WV, Aman MJ, Bavari S. Ebola virus-like particle-based vaccine protects nonhuman primates against lethal Ebola virus challenge. *J Infect Dis*. 2007;196(Suppl 2):S430–S437. (2) Bukreyev A, Rollin PE, Tate MK, et al. Successful topical respiratory tract immunization of primates against Ebola virus. *J Virol*. 2007;81:6379–6388. (3) Hensley LE, Mulangu S, Asiedu C, et al. Demonstration of cross-protective vaccine immunity against an emerging pathogenic ebolavirus Species. *PLoS Pathog*. 2010;6:e1000904. (4) Sullivan NJ, Sanchez A, Rollin PE, Yang ZY, Nabel GJ. Development of a preventive vaccine for Ebola virus infection in primates. *Nature*. 2000;408:605–609. (5) Blaney JE, Marzi A, Willet M, et al. Antibody quality and protection from lethal Ebola virus challenge in nonhuman primates immunized with rabies virus based bivalent vaccine. *PLoS Pathog*. 2013;9:e1003389. (6) Stanley DA, Honko AN, Asiedu C, et al. Chimpanzee adenovirus vaccine generates acute and durable protective immunity against ebolavirus challenge. *Nat Med*. 2014;20:1126–1129. (7) Swenson DL, Wang D, Luo M, et al. Vaccine to confer to nonhuman primates complete protection against multistrain Ebola and Marburg virus infections. *Clin Vaccine Immunol*. 2008;15:460–467. (8) Sullivan NJ, Geisbert TW, Geisbert JB, et al. Accelerated vaccination for Ebola virus haemorrhagic fever in non-human primates. *Nature*. 2003;424:681–684. (9) Sullivan NJ, Geisbert TW, Geisbert JB, et al. Immune protection of nonhuman primates against Ebola virus with single low-dose adenovirus vectors encoding modified GPs. *PLoS Med*. 2006;3:e177. (10) Herbert AS, Kuehne AI, Barth JF, et al. Venezuelan equine encephalitis virus replicon particle vaccine protects nonhuman primates from intramuscular and aerosol challenge with ebolavirus. *J Virol*. 2013;87:4952–4964. (11) Geisbert TW, Daddario-Dicaprio KM, Geisbert JB, et al. Vesicular stomatitis virus-based vaccines protect nonhuman primates against aerosol challenge with Ebola and Marburg viruses. *Vaccine*. 2008;26:6894–6900. (12) Jones SM, Feldmann H, Ströher U, et al. Live attenuated recombinant vaccine protects nonhuman primates against Ebola and Marburg viruses. *Nat Med*. 2005;11:786–790. (13) Daddario-DiCaprio KM, Geisbert TW, Geisbert JB, et al. Cross-protection against Marburg virus strains by using a live, attenuated recombinant vaccine. *J Virol*. 2006;80:9659–9666. (14) Mire CE, Geisbert JB, Agans KN, et al. Durability of a vesicular stomatitis virus-based Marburg virus vaccine in nonhuman primates. *PLoS ONE*. 2014;9:e94355. (15) Mire CE, Geisbert JB, Marzi A, Agans KN, Feldmann H, Geisbert TW. Vesicular stomatitis virus-based vaccines protect nonhuman primates against Bundibugyo ebolavirus. *PLoS Negl Trop Dis*. 2013;7:e2600. (16) Falzarano D, Feldmann F, Grolla A, et al. Single immunization with a monovalent vesicular stomatitis virus-based vaccine protects nonhuman primates against heterologous challenge with Bundibugyo ebolavirus. *J Infect Dis*. 2011;204(suppl 3):S1082–S1089. (17) Geisbert TW, Geisbert JB, Leung A, et al. Single-injection vaccine protects nonhuman primates against infection with Marburg virus and three species of Ebola virus. *J Virol*. 2009;83:7296–7304.

the secreted proteins $GP_{1,2\Delta}$, 34 sGP,[211] ssGP,[33] and Δ-peptide.[212,213] Although the function of these molecules is unclear, large concentrations of at least sGP in the serum of infected animals suggest that they might interfere with the host immune response by, for instance, serving as decoys for anti-$GP_{1,2}$ antibodies.[211]

Once inside the cell, several filovirus proteins actively suppress the innate cellular immune response.[214] VP35 protects double-stranded viral RNA intermediates produced during genome replication to prevent recognition by the host pattern recognition receptors melanoma-differentiation-associated protein-5 and retinoic-acid-inducible protein-1. Filovirus VP35 is also a powerful host cell RNA silencing suppressor,[215] and $GP_{1,2}$ antagonizes the cellular viral restriction factor tetherin.[216] In addition, VP35 inhibits interferon response factor 3 and 7 phosphorylation and inhibits interferon (IFN) α/β production.[217–219] Ebolavirus VP24 prevents karyopherin shuttling from the cytoplasm into the nucleus and thus inhibits host cell signaling downstream of IFN-α/β/γ.[220–222] In the case of marburgviruses, VP40—but not VP24—interferes with the IFN pathway. MARV VP40 inhibits signal transducer and activator transcription phosphorylation in response to type I and II IFN and interleukin-6 (IL-6).[223]

The sequence of events during MVD and EVD pathogenesis is likely determined by the accessibility of susceptible cell types for filovirions, the route of infection, and the responses of these cells to infection. Sessile and mobile cells of the mononuclear phagocytic system (alveolar, peritoneal, pleural macrophages; Kupffer cells; microglia) and dendritic cells are initially infected.[20,102,187,224–229] Filoviruses then spread via the lymphatics to regional lymph nodes and via blood to the liver, spleen, and other organs.[224,228,230]

In fundamental ways, macrophages and dendritic cells react differently to filovirus infections. Macrophages are activated upon infection and react with the release of proinflammatory cytokines, such as IL-1β, IL-6, and IL-8, and tumor necrosis factor-α (TNF-α), while releasing progeny virions.[208,227,231–236] This release results in a recruitment of additional macrophages to the infection site, resulting in a vicious cycle of infection of additional macrophages. Increasing amounts of virions are released and spread through the blood, and they are measurable as increased viremia.[224] Dendritic cells, however, react with aberrant responses to infection. Major histocompatibility complex class II is partially suppressed, and expression of tissue factor and the TNF-related apoptosis-inducing ligand is increased.[237,238]

Together, these responses may be the cause of observed death of bystander lymphocytes and general lymphoid hypoplasia in lymph nodes, spleen, and thymus. Lymphoid hypoplasia with the inhibition of IFN pathways at least partially explains the pronounced immunosuppression observed in people with fatal infections.[239,240]

The combined effects of the initial events in filovirus infection probably lead to broad organ system dysregulation, exposure of previously shielded filovirus-susceptible cells, or the transformation of resistant to susceptible cells. Endothelial cells are activated through filovirus infection-induced cytokines.[241] This activation is marked by increased expression and/or release of intravascular adhesion molecule-1, vascular cell adhesion molecule-1, and E- and P-selectin. Consequently, breakdown of the endothelial barrier function results from induced changes in the cadherin/catenin composition of adherens junctions in vascular endothelial cells.[230,234,242–244] The result of this dysregulation is probably massive fluid redistribution (third spacing), which is evidenced clinically by widespread edema and possibly hypovolemic shock. Later in infection, endothelial cells, adrenal cortical cells, epithelial cells, reticular fibroblasts, hepatocytes, and reproductive cells among others also become directly infected with filoviruses, leading to cytolytic infection.[20,187,208,224,245–248]

The increasing concentrations of proinflammatory cytokines (IL-1, IL-6, IL-8, TNF-α, MCP-1, MIP-1α) and other mediators, such as tissue factor, probably form the basis for the induction of disseminated intravascular coagulation (DIC).[249–253] The destruction of adrenal cortical cells results in decreased steroid synthesis, leading to hypotension and therefore further stasis in blood vessels, which may fuel DIC.[208] Consequently, numerous microthrombi form in the vascular system and occlude smaller blood vessels especially. Hypoxemic infarcts develop in downstream tissue, manifested as multiple focal necroses in gonads, kidneys, liver, spleen, and other organs. The continuous destruction of the liver, clinically measurable as an increase in liver enzyme concentrations, leads to a dramatic decrease in albumin and clotting factors. Decreased albumin leads to further fluid redistribution (edema). DIC ceases once all circulating clotting factors have been consumed and leads to petechial rashes, ecchymoses, and general uncontrolled (but rarely life-threatening) hemorrhages.[187,246,254,255] Multiple organ dysfunction syndrome frequently occurs as a result of this series of events.

Clinical Presentation

MVD and EVD cannot be distinguished on grounds of clinical observation alone. Based on the few larger cohort studies published, few to no statistically significant differences were noted in the onset, duration, frequency,

or type of clinical signs and symptoms of MVD and EVD caused by the six filoviruses. Clinical presentation is overall dynamic, with most "typical" signs occurring in many—but not all—cases of infection.[171–177]

The current understanding of human filovirus disease is based largely on observations made during the MVD and EVD outbreaks resulting from MARV infection in Frankfurt/Main and Marburg in West Germany in 1967[71,192,256–259] and in Durba and Watsa in the Democratic Republic of the Congo in 1998–2000[174]; EVD outbreaks resulting from EBOV infection around Yambuku, Zaire, 1976,[260,261] Kikwit, Zaire, 1995,[171] Boene (Democratic Republic of the Congo),[178] and in Western Africa, 2013–2015[172,173,262]; an EVD outbreak resulting from SUDV infection around Yambio, Sudan, 1976[263]; an EVD outbreak resulting from BDBV infection in Bundibugyo, Uganda, 2007[121,175] (Table 23-5); and single-case exportations or laboratory accidents. The incubation period of filovirus disease is highly variable (3–25 days, probably dependent on the route of infection and the amount of virus transmitted). Phase 1, the prodromic phase of the disease that coincides with virema, lasts 5 to 7 days and generally resembles influenza. This phase is characterized by a sudden onset of fever (>38.6°C) and chills, abdominal pain, arthralgia, cough, chest pain and shortness of breath, severe headaches, myalgia, pharyngitis, and the appearance of a morbilliform/maculopapular rash (which, however, may be difficult to see on black skin).

Phase 2 begins after 1 to 2 days of relative remission with a more dramatic clinical presentation including almost all organ systems. Severe abdominal pain, vomiting, and watery diarrhea mark the gastrointestinal effects of infection. Confusion, tremors, psychosis, and coma demonstrate the involvement of the central nervous system. Edema and orthostatic hypotension are both primary and secondary effects of filovirus replication in a variety of cells in the liver, adrenal glands, and vascular system. Induced DIC followed by a total lack of coagulative responses lead to hemorrhagic manifestations. Hemorrhage, which appears in only about 50% of the cases, includes bleeding from mucosal surfaces (gums, nose, rectum, vagina) and venipuncture sites, resulting in detectable blood in sputum, feces, urine, and vomit. Other hemorrhagic manifestations are subconjunctival hemorrhage, petechiae, purpura, and ecchymoses.[171,191,233,238,251,256,257,264–267] On palpation, hepatomegaly is usually prominent, but jaundice is typically absent. Given the filovirus-induced immunosuppression, secondary bacterial and/or fungal infections may develop.

Death is usually the result of multiple organ dysfunction syndrome. Although multiple organ dysfunction syndrome may be caused by fluid redistribution (third spacing) and the multiple necroses in organs, death resulting from blood loss is extremely rare and occurs most frequently among women in labor.[268–270] Survival of EVD, which is inversely correlated with viremia, is associated with particular immunoglobulin M and immunoglobulin G responses and a strong proinflammatory response early in the course of disease,[249,250,252,271–273] and it is probably influenced by host genetics.[274] Survivors of MVD and EVD may experience a wide variety of long-term sequelae that include amnesia, anxiety, joint pain, skin peeling and hair loss, fatigue, hepatitis, myalgia, myelitis, ocular manifestations (choroiditis, iridocyclitis, iritis, uveitis), hearing loss, orchitis, and/or psychosis. At least three filoviruses, MARV, EBOV, and SUDV, may induce persistent infections in the liver, eye, or gonads beyond reconvalescence, and may later reactivate or be transmitted sexually.[120,275–280]

Typical clinical laboratory parameters of MVD and EVD are progressing leukopenia (as low as 1,000/μl) caused by the loss of lymphocytes with a left shift followed by leukocytosis resulting from an increase in granulocytes, and mild thrombocytopenia (50,000–100,000/μl). Liver, kidney, and pancreas dysfunction are evident in the form of increased concentrations of aspartate aminotransferase, alanine aminotransferase, γ-glutamyltransferase, amylase, creatinine, and urea, as well as hypokalemia. The effect of filovirus infection on the coagulation cascade becomes evident via prolonged prothrombin time and partial thromboplastin time and increased D-dimer concentrations.[84,171–173,189,191,257,261,281–283]

DIAGNOSIS

MVD or EVD should be considered in any acutely febrile patient who resides or has travelled through a filovirus-endemic area. A history of rural travel, expeditions into the rain forest or natural or artificial caves, and contact with sick or deceased animals, including humans, should raise suspicion. However, as the recent 2013–2015 EVD outbreak in Western Africa demonstrated, filoviruses may be more broadly distributed than previously thought. Thus, the possibility of filovirus infection in a febrile patient from an African country without recorded filovirus infection should not be discounted. Unfortunately, MVD and EVD patients present with rather unspecific, influenza-like clinical signs caused by numerous pathogens that are more commonly encountered. Even later stages of MVD and EVD are easily confused with the clinical presentation of other diseases (Table 23-6).

TABLE 23-5

CLINICAL PRESENTATION OF MARBURG VIRUS DISEASE OR EBOLA VIRUS DISEASE (ADAPTED AND AVERAGED FROM DATA SOURCES)

Clinical Signs and Symptoms in Humans	Survivors of MARV Infection (%)	Fatal MARV Infections (%)	Survivors of BDBV Infection (%)	Fatal BDBV Infections (%)	Survivors of EBOV Infection (%)	Fatal EBOV Infections (%)
Abdominal pain	59	57	73	88	27	26
Anorexia/appetite loss	77	72	68	77		
Anuria			13	18		
Arthralgia or myalgia	55	55	74	80	25	51
Asthenia			73	82	13	61
Bleeding from the gums	23	36		9		
Bleeding from any site	59	71	29	54	22	47
Bleeding from GI tract				6	19	
Chest pain	18	4	13	45		
Confusion/disorientation			27	36		19
Conjunctival injection/ conjunctivitis	14	42	47	55	13	
Cough	9	5	7	36	13	17
Diarrhea	59	56	83	92	27	44
Difficulty breathing/ distress	36	58	18	88	8	23
Dizziness					13	56
Epistaxis	18	34	7	9		
Facial/neck edema		92		82		24
Fever	10	29	78	81	71	74
Headaches	73	79	82	85	57	46
Hematemesis	68	76		18		
Hemoptysis	9	4		9		
Hiccups	18	44	18	40	2	13
Lumbar pain	5	8	5	36		
Maculopapular rash/ rash			25	27		
Malaise or fatigue	86	83	56	100		
Melena	41	58		27		
Nausea and vomiting	77	76	76	88	59	62
Petechiae	9	7				3
Sore throat, odynophagia, or dysphagia	43	43	45	50	13	31

See Figure 23-1 for color explanations.
BDBV: Bundibugyo virus
EBOV: Ebola virus
GI: gastrointestinal
MARV: Marburg virus
Data sources: (1) Bwaka MA, Bonnet MJ, Calain P, et al. Ebola hemorrhagic fever in Kikwit, Democratic Republic of the Congo: clinical observations in 103 patients. *J Infect Dis*. 1999;179(Suppl 1):S1–S7. (2) Bah EI, Lamah MC, Fletcher T, et al. Clinical presentation of patients with Ebola virus disease in Conakry, Guinea. *N Engl J Med*. 2015;372:40–47. (3) Schieffelin JS, Shaffer JG, Goba A, et al. Clinical illness and outcomes in patients with Ebola in Sierra Leone. *N Engl J Med*. 2014;371:2092–2100. (4) Bausch DG, Nichol ST, Muyembe-Tamfum JJ, et al. Marburg hemorrhagic fever associated with multiple genetic lineages of virus. *N Engl J Med*. 2006;355:909–919. (5) MacNeil A, Farnon EC, Wamala J, et al. Proportion of deaths and clinical features in Bundibugyo Ebola virus infection, Uganda. *Emerg Infect Dis*. 2010;16:1969–1972. (6) Roddy P, Howard N, Van Kerkhove MD, et al. Clinical manifestations and case management of Ebola haemorrhagic fever caused by a newly identified virus strain, Bundibugyo, Uganda, 2007–2008. *PLoS One*. 2012;7:e52986. (7) Barry M, Traore FA, Sako FB, et al. Ebola outbreak in Conakry, Guinea: epidemiological, clinical, and outcome features. *Med Mal Infect*. 2014;44:491–494.

Once filovirus infection is suspected, it is imperative to contact the proper public health authorities and infectious disease specialists and to perform all patient contact and sample handling with utmost caution.[284] Filovirus infection can be confirmed safely and relatively easily in mobile field laboratories, local hospitals, and/or reference laboratories as long as the necessary technology and trained staff are available. Reverse

TABLE 23-6

MARBURG VIRUS DISEASE AND EBOLA VIRUS DISEASE DIFFERENTIAL DIAGNOSIS (ADAPTED FROM DATA SOURCES)

Viral Infections	Bacterial Infections	Fungal Infections	Parasite Infections	Noninfectious Diseases
Major: fulminant viral hepatitides; measles; rubella; VHFs caused by Lassa virus or yellow fever virus	Major: Enterohemorrhagic *Escherichia coli* enteritis; gram-negative bacterial septicemia; leptospirosis; murine typhus; rickettsial diseases; shigellosis; typhoid fever; typhus	Major: histoplasmosis	Major: falciparum malaria	Major: acute promyelocytic leukemia; factor VII, IX, and X deficiencies; hemolytic uremic syndrome; hereditary hemorrhagic telangiectasia; Kawasaki disease; platelet and vascular disorders; snake envenomation; thrombotic thrombocytopenic purpura; warfarin intoxication
Minor: chikungunya, hepatitis A, B, non-A/B; herpes simplex; influenza; mononucleosis; Sindbis disease; West Nile virus fever; VHFs caused by other viruses	Minor: anthrax; bartonellosis; campylobacteriosis; meningococcal septicemia; plague; *Pseudomonas* infections; psittacosis with endocarditis; Q fever; relapsing fever; staphylococcal septicemia; streptococcal septicemia/rheumatic fever	Minor: candidiasis	Minor: trypanosomiasis, visceral leishmaniasis	Minor: drug rashes

VHF: viral hemorrhagic fever

Data sources: (1) Kuhn JH. *Filoviruses. A Compendium of 40 Years of Epidemiological, Clinical, and Laboratory Studies.* In: Carlisher CH, ed. *Archives of Virology* Supplementa Series, Vol 20. Vienna, Austria: Springer-Verlag Wien; 2008. (2) Grolla A, Lucht A, Dick D, Strong JE, Feldmann H. Laboratory diagnosis of Ebola and Marburg hemorrhagic fever. *Bull Soc Pathol Exot*. 2005;98:205–209. (3) Boisen ML, Schieffelin JS, Goba A, et al. Multiple circulating infections can mimic the early stages of viral hemorrhagic fevers and possible human exposure to filoviruses in Sierra Leone prior to the 2014 outbreak. *Viral Immunol*. 2015;28:19–31.

transcriptase-PCR is the method of choice for detection of filovirus genomes (detection limit: ≈1,000–2000 genome copies/ml of serum) in, for instance, guanidinium isothiocyanate-inactivated samples.[285–287] The less sensitive antigen-capture ELISA and antibody-capture ELISA are alternative or complementary assays for the detection of filovirus proteins and anti-filovirus antibodies in 60Co-irradiated samples.[288,289] Samples from skin biopsies can be inactivated by formalin fixation and then used to diagnose filovirus infection using immunohistochemistry or in situ hybridization.[102,290]

Noninactivated samples, such as acute-phase serum or blood which typically contain high filovirus titers and antifilovirus antibodies,[291] must not be handled outside of a maximum-containment (biosafety level 4) laboratory. Such samples should be collected with utmost caution using proper PPE and then sent to the appropriate World Health Organization reference laboratories using suitable transport media. Filoviruses typically grow quickly and to high titers in standard cell cultures such as grivet Vero E6, rhesus monkey MA-104, or human adrenal carcinoma SW-133 cells.[292,293] From these infected cells, additional studies can be performed, such as variant isolation and typing, sample virus quantification by plaque assays, standard (consensus) Sanger genome sequencing, and easy visualization of typical shapes of filovirions using electron microscopy.[294,295] An overview of current diagnostic options is provided in Table 23-7.

TABLE 23-7

LABORATORY DIAGNOSIS OF FILOVIRUS INFECTION (ADAPTED FROM DATA SOURCES)

PRIMARY ASSAYS

Diagnostic Test	Target	Clinical Material	Advantage	Disadvantage
RT-PCR	Filovirus subgenomic, genomic, or antigenomic nucleic acids	Blood, serum, tissue	Rapid; ultra-sensitive; specific; can be performed on inactivated samples	Requires PCR machine; laboratory cross-contamination can lead to false-positive results; release of RT-PCR inhibitors from tissue can lead to false negative results
Antigen-capture ELISA	Filovirus antigen/ proteins	Blood, serum, tissue possible	Rapid; sensitive; specific; can be performed on inactivated samples; high-throughput	Requires ELISA reader
IgG-capture ELISA	Antifilovirus antibodies (late in infection; survivors)	Serum	Rapid; sensitive; specific; can be performed on inactivated samples	Requires ELISA reader and large amounts of purified native or recombinant filoviral antigen; some patients do not seroconvert
IgM-capture ELISA	Antifilovirus antibodies (early in infection)	Serum	Rapid; sensitive; specific; can be performed on inactivated samples	Requires ELISA reader and large amounts of purified native or recombinant filoviral antigen; some patients do not seroconvert

SECONDARY/CONFIRMATORY ASSAYS

Diagnostic Test	Target	Source	Advantage	Disadvantage
Virus isolation	Filoviruses	Blood, tissue	Specific	Requires maximum-containment laboratory and time; filovirus isolation may fail or filovirus replication may not cause CPE in cell cultures during initial passages
Electron microscopy	Complete or fragmented filovirions or characteristic cellular inclusion bodies	Blood, serum, tissue	Specific	Insensitive; requires electron microscope
Indirect immuno-fluorescent assay	Antifilovirus antibodies	Serum	Rapid; simple; safe	Insensitive; possible cross reactions leading to false positive results; subjective interpretation; some patients do not seroconvert
Fluorescent assay	Filovirus antigen/ proteins	Tissue culture/ isolated virus	Rapid; simple; safe	Insensitive; requires infectious material and specific antibodies; subjective interpretation
Next-generation sequencing	Filovirus subgenomic, genomic, or antigenomic nucleic acids	Blood, serum, tissue	Very specific; ultra-sensitive; can determine coding-complete filovirus genomes in absence of virus culture; allows molecular epidemiology	New and expensive technology; not yet widespread; requires highly trained personnel and bioinformatics support
Immunohisto-chemistry	Filoviral antigen	Tissue (skin, liver)	Tissue can be fixed	Requires time and specific antibodies

(**Table 23-7** *continues*)

Table 23-7 *continued*

In situ hybridization	Filoviral nucleic acids	Tissue	Tissue can be fixed	Requires special equipment and specific probes
Western blot	Antifilovirus antibodies	Serum	Specific	Difficult interpretation; requires specific antibodies

CPE: cytopathic effect
ELISA: enzyme-linked immunosorbent assay
IgG: immunoglobulin G
IgM: immunoglobulin M
RT-PCR: reverse transcriptase polymerase chain reaction
Data sources: (1) Kuhn JH. *Filoviruses: A Compendium of 40 Years of Epidemiological, Clinical, and Laboratory Studies*. In: Calisher CH, ed. *Archives of Virology* Supplementa Series, Vol 20. Vienna, Austria: Springer-Verlag Wien; 2008. (2) Gire SK, Goba A, Andersen KG, et al. Genomic surveillance elucidates Ebola virus origin and transmission during the 2014 outbreak. *Science*. 2014;345:1369–1372. (3) Grolla A, Lucht A, Dick D, Strong JE, Feldmann H. Laboratory diagnosis of Ebola and Marburg hemorrhagic fever. *Bull Soc Pathol Exot*. 2005;98:205–209. (4) Wang YP, Zhang XE, Wei HP. Laboratory detection and diagnosis of filoviruses. *Virol Sin*. 2011;26:73–80.

TREATMENT

Treatment of MVD and EVD patients is challenging because of the healthcare workers' risk of infection. Patients should be isolated, infection control precautions/strict barrier-nursing techniques need to be implemented, and healthcare personnel must wear proper PPE. In addition, standard operating procedures should be in place for safe clinical sample management; decontamination of possibly contaminated tools, materials or surface equipment and personnel; point-of-care laboratory testing; and infectious waste management.

No FDA-approved specific therapy is available to treat human infections. Although statistical reports are still lacking, chances for survival from MVD and EVD are now thought to be dramatically increased through aggressive supportive therapy.[262,296,297] Treatment should follow the guidelines for severe sepsis management and therefore aim to reestablish fluid and electrolyte balance and reversal of DIC, hemorrhage, hypotension/hypoperfusion, acute kidney injury, and shock. As filoviruses potently suppress the immune system, empirical and possibly prophylactic treatment of secondary bacterial and/or fungal infections with broad-spectrum antibiotics (eg, vancomycin, piperacillin/tazobactam) and antimycotics is advised. Pain management and administration of antiemetics should always be considered.[180,181,262]

Numerous drugs have been evaluated in vitro and in various animal models over the years to identify candidate MCMs to treat MVD and EVD.[298–302] Although none of them has reached medical licensure, several have been sufficiently promising for emergency use in humans. An overview of the most commonly discussed MCMs for filovirus disease treatment is provided in Table 23-8.

SUMMARY

Due to extremely low human case numbers, filovirus infections were long considered exotic infectious diseases of no larger consequence to global public health. The still ongoing EVD outbreak in Western Africa, which by now includes close to 28,500 cases and 11,300 deaths, brought awareness to the fact that single filovirus introductions into the human population may lead to devastating and large epidemics that can spread quickly across international borders. Unfortunately, despite considerable scientific progress, many key questions regarding filoviruses remain to be answered. First, and foremost, the natural filovirus host reservoirs have to be identified so that preventive measures against initial zoonotic spillover into humans can be established. Second, almost all filovirus research currently focuses only on EBOV, SUDV, and MARV, while next to nothing is known about the molecular or pathogenic features of other filoviruses. Yet, medical countermeasures should be created that can be used against any human (and possibly) animal infection because it is currently unpredictable which filovirus will strike next.

TABLE 23-8

SELECTED PROMISING CANDIDATE THERAPEUTICS FOR FILOVIRUS INFECTIONS

Candidate MCM	Mechanism of Action	Efficacy	Additional Information
ZMapp (Mapp Biopharmaceutical, Inc/LeafBio, Inc)[1]	Cocktail of three monoclonal antibodies (c13C6, c2G4, c4G7) targeting **EBOV** $GP_{1,2}$	100% survival of rhesus monkeys up to 5 days after **EBOV** exposure	Produced in genetically modified tobacco plants; may not be scalable; requires frozen shipping. Currently in phase 1/2 clinical trials
MB-003 (USAMRIID/ Mapp Biopharmaceutical)[2,3]	Cocktail of three monoclonal antibodies (c13C6, h-13F6, c6D8) targeting **EBOV** $GP_{1,2}$	100% survival of rhesus monkeys 1 h after **EBOV** exposure; 67% and 43% survival at 2 days and 4–5 days after exposure, respectively	Produced in genetically modified tobacco plants; scalable; requires frozen shipping
ZMAb (PHAC/Defyrus Inc)[4–7]	Cocktail of three monoclonal antibodies (1H3, 2G4, 4G7) targeting **EBOV** $GP_{1,2}$	100% survival of crab-eating macaques 1 day after **EBOV** exposure; 50% survival at 2 days after exposure. 100% survival of survivors after reexposure to **EBOV** 10 weeks after challenge, 67% survival after 13 weeks	Requires frozen shipping
Immucillin-A/BCX4430 (BioCryst Pharmaceuticals)[8]	Nucleoside analog that inhibits the **MARV** RNA-dependent RNA polymerase and causes lethal mutagenesis	100% survival of crab-eating macaques 48 h after **MARV** exposure	In phase 1 clinical trial
Favipiravir/T-705 (Fujifilm/Toyama Chemical Co, Ltd)[9,10]	Nucleotide analog that inhibits the filovirus RNA-dependent RNA polymerase and causes lethal mutagenesis	100% survival of IFNAR-/- laboratory mice 6 days after parenteral mouse-adapted **EBOV** exposure; 17% survival of rhesus macaques	Used as a licensed antiinfluenza drug in Japan. Contraindicated in pregnancy because of possibility of teratogenicity and embryotoxicity. In phase 3 clinical trials (FLUAV). Currently is being evaluated on EVD patients in Guinea in a single-arm phase 2 clinical trial
JK-05 (Sihuan Pharmaceutical Holdings Group, Ltd and Academy of Military Medical Sciences)[11]	Nucleotide analog that inhibits the filovirus RNA-dependent RNA polymerase and causes lethal mutagenesis	Efficacy in laboratory mice	Considered for use in emergency situations
TKM-Ebola/Tekmira-100802 (Tekmira Pharmaceuticals Corp)[12,13]	Lipid nanoparticle cocktail of siRNAs targeting **EBOV** VP35, VP24, and L	100% survival of rhesus monkeys 30–60 min after **EBOV** exposure; 83%, 50%, and 67% survival at 1, 2, and 3 days after exposure, respectively	Phase 1 clinical trial aborted
"TKM-Marburg" (Tekmira Pharmaceuticals Corp)[14]	Lipid nanoparticle cocktail of siRNAs targeting **MARV** NP	100% survival of rhesus monkeys 30–45 min, 1 day, 2 days, and 3 days after **MARV** exposure	
AVI-7537 (Sarepta Therapeutics)[15]	Phosphorodiamidate morpholino oligomer targeting **EBOV** VP24	63% survival of rhesus monkeys 1 h after **EBOV** exposure	Phase 1 clinical trial

(**Table 23-8** *continues*)

Table 23-8 *continued*

AVI-6002 (Sarepta Therapeutics)[16]	Phosphorodiamidate morpholino oligomer targeting **EBOV** VP35 and VP24	>60% survival of rhesus monkeys 30–60 min after **EBOV** exposure	Phase 1 clinical trial completed
rVS[I]VΔG-ZEBOV-GP/ BPSC1001 (Newlink Genetics/PHAC)[17]	Postexposure vaccine consisting of a recombinant replicating vesicular stomatitis Indiana virus expressing **EBOV** $GP_{1,2}$ to stimulate anti-$GP_{1,2}$ immune responses	50% survival of rhesus monkeys 20–30 min after **EBOV** exposure; 100% and 50% survival of laboratory mice and guinea pigs, respectively, 24 h after **EBOV** exposure	Easy to produce; requires frozen shipping; concerns about immunocompromised patients. Currently in phase 1 clinical trials
Other rVS[I]V formulations[18–20]	Postexposure vaccine consisting of a recombinant replicating vesicular stomatitis Indiana virus expressing filovirus $GP_{1,2}$ to stimulate anti-$GP_{1,2}$ immune responses	**MARV**: 100% survival of rhesus monkeys 20–30 min after **MARV** exposure; 83% and 33% survival at 1 day and 2 days after exposure, respectively SUDV: 100% survival of rhesus monkeys 20–30 min after **SUDV** exposure	Easy to produce; requires frozen shipping; concerns about immunocompromised patients
Recombinant Nematode Anticoagulant Protein c2 (rNAPc2)[21]	Inhibits factor VIIa/tissue factor complex and blood clot formation	33% survival of rhesus monkeys 10 min and 1 day after **EBOV** exposure	In phase 2 clinical trial for second-line treatment of metastatic colorectal carcinoma in combination with contemporary 5-FU-based chemotherapy
Activated drotrecogin alfa/Xigris (Eli Lilly and Company)[22]	Recombinant human activated protein C; inhibits coagulation factors Va and VIIIa (antithrombotic)	20% survival of rhesus monkeys 1 h after **EBOV** exposure	Withdrawn from market
Hyperimmune equine[23,24] immunoglobulin G[23–25]	Filovirions	50%–100% survival of hamadryas baboons 5–15 min after **EBOV** exposure; 80%, 20%–100%, and 29% survival at 30 min, 1 h, and 2 h after exposure, respectively	Licensed in Russia for treatment of occupational accidents. Highly immunogenic in humans. Evaluation in rhesus monkeys (using different dose and virus variant) not successful
Passive transfer of convalescent or postimmunization plasma[26–28]	Filovirions	Passive transfer of concentrated polyclonal IgG from immune rhesus monkeys resulted in 100% survival of rhesus monkeys at 15–30 min and 48 h after **MARV** exposure	Uncontrolled experiment during the 1995 EVD/**EBOV** outbreak in Kikwit, Zaire, suggested passive transfer of whole blood to be protective for 7 of 8 patients

See Figure 23-1 for color explanations.
ADE: adverse effects
ADV: adenovirus
CMV: cytomegalovirus
EBOV: Ebola virus
EVD: Ebola virus disease
FLUAV: influenza A virus
5-FU: fluorouracil
GP: glycoprotein
IFN: interferon
IFNAR: interferon-α/β receptor
IgG: immunoglobulin G
MARV: Marburg virus
MCM: medical countermeasures
PHAC: Public Health Agency of Canada
siRNA: short interfering RNA
SUDV: Sudan virus
USAMRIID: US Army Medical Research Institute of Infectious Diseases
VP: viral protein

(**Table 23-8** *continues*)

Table 23-8 *continued*

Note: Information on the status of all ongoing filovirus-relevant clinical trials can be found at https://ClinicalTrials.gov with the search terms "Ebola" or "Marburg."

Data sources: (1) Qiu X, Wong G, Audet J, et al. Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. *Nature*. 2014;514:47–53. (2) Shurtleff AC, Biggins JE, Keeney AE, et al. Standardization of the filovirus plaque assay for use in preclinical studies. *Viruses*. 2012;4:3511–3530. (3) Pettitt J, Zeitlin L, Kim do H, et al. Therapeutic intervention of Ebola virus infection in rhesus macaques with the MB-003 monoclonal antibody cocktail. *Sci Transl Med*. 2013;5:199ra113. (4) Qiu X, Audet J, Wong G, et al. Successful treatment of Ebola virus-infected cynomolgus macaques with monoclonal antibodies. *Sci Transl Med*. 2012;4:138ra181. (5) Audet J, Wong G, Wang H, et al. Molecular characterization of the monoclonal antibodies composing ZMAb: a protective cocktail against Ebola virus. *Sci Rep*. 2014;4:6881.(6) Qiu X, Audet J, Wong G, et al. 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Acknowledgments

We are grateful for the technical writing services offered by Laura Bollinger (IRF-Frederick) and the support by IRF-Frederick's medical illustrator, Jiro Wada. The content of this publication does not necessarily reflect the views or policies of the US Department of Defense, US Department of Health and Human Services, the US Department of the Army, or of the institutions and companies affiliated with the authors. This work was funded in part through Battelle Memorial Institute's prime contract with the US National Institute of Allergy and Infectious Diseases under Contract No. HHSN272200700016I. A subcontractor to Battelle Memorial Institute who performed this work is: JHK, an employee of Tunnell Government Services, Inc.

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Chapter 24

SMALLPOX AND RELATED ORTHOPOXVIRUSES

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SUMMARY

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INTRODUCTION

Variola virus, the causative agent of smallpox, is one of the most significant bioterrorist threat agents. During the 20th century, smallpox was estimated to have caused more than 500 million human deaths.[1] The disease and the naturally circulating virus itself were eradicated by the World Health Organization's (WHO) global eradication campaign, which was declared a success in 1980.[2] This program, which involved vaccinating all humans in a ring surrounding every suspected case of variola infection, was successful in part because smallpox is solely a human disease with no animal reservoirs to reintroduce the virus into the human population. The impact of a potential smallpox virus attack in the human population would be more catastrophic now than in previous outbreaks. The pace of viral spread would be accelerated since most vaccination programs were abandoned worldwide in the 1970s, the prevalence of immune-suppressed individuals with human immunodeficiency virus (HIV) infections and those undergoing chemotherapy for various cancers have grown, and human mobility including intercontinental air travel has increased.

Variola virus is stable, highly infectious via the aerosol route, highly transmissible from infected to susceptible persons, and has a relatively long asymptomatic incubation period making contact tracing difficult.[3] Mathematical models of the reintroduction of variola into contemporary human populations indicate dire consequences.[4] Public health experts have argued that a significant portion of the population should be prevaccinated to blunt the impact of a potential attack.[5] However, the current licensed vaccine is associated with significant adverse events,[6] which are more serious in persons who are immunocompromised, and prerelease vaccination is contraindicated for a significant portion of the population.

Revelations in the 1990s that the former Soviet Union produced ton quantities of variola virus as a strategic weapon and conducted open air testing of aerosolized variola on Vozrozhdeniya Island in the Aral Sea have increased the plausibility of variola being used as a bioterrorism agent.[3,7] Considerable investment is being made in biopreparedness measures against smallpox and related orthopoxviruses, including emergency response plans for mass immunization and quarantine, as well as development of improved countermeasures such as new vaccines and antiviral drugs.[8,9] These countermeasures are also needed to respond to the public health threat of the closely related monkeypox virus, which occurs naturally in western and central Africa and produces a disease in humans that closely resembles smallpox. Alibek claimed that the monkeypox virus was weaponized by the former Soviet Union.[10] The monkeypox virus was first isolated from cynomolgus macaques in Denmark in 1958; however, scientific interest did not increase until the 1970s when it was demonstrated that monkeypox virus can cause lethal infection in humans.[11,12] A dramatic increase in monkeypox virus incidence has occurred in the Democratic Republic of the Congo (DRC) over the past 30 years following the cessation of routine smallpox vaccination, and current estimates state that approximately 50% of the DRC general population is not protected against either the monkeypox virus or variola virus.[13] Monkeypox virus was imported inadvertently into the United States in 2003 via a shipment of rodents originating in Ghana, where, in contrast to the significant morbidity and mortality seen in the DRC, little morbidity was associated with infection.[14,15] Consequently, more than 50 human infections were documented in the United States, thus demonstrating the public health importance of this agent and its potential bioterrorist threat.[14,15]

AGENT CHARACTERISTICS

Classification

Poxviruses infect most vertebrates and invertebrates, causing various diseases of veterinary and medical importance. The poxvirus family is divided into two main subfamilies: (1) the *Chordopoxvirinae*, which infects vertebrates; and (2) the *Entomopoxvirinae*, which infects insects. Subfamily *Chordopoxvirinae* is divided into eight genera, one of which is *Orthopoxvirus*, which consists of numerous genetically similar pathogens capable of causing disease in humans including variola virus (Figure 24-1), monkeypox virus, cowpox virus, and vaccinia virus.[16] Members of the *Orthopoxvirus* genus are mostly zoonotic pathogens (Table 24-1) that are antigenically similar.[17] Antigenic similarity was vital to the smallpox eradication in 1980 and remains a key component for the protection of military personnel, healthcare workers, and researchers who are likely to come into contact with orthopoxviruses.[9,18]

Morphology

Orthopoxviruses are oval, brick-shaped particles with a geometrically corrugated outer surface (Figure 24-2). Their size ranges from 220 nm to 450 nm long and 140 nm to 260 nm wide.[18] The outer envelope consists

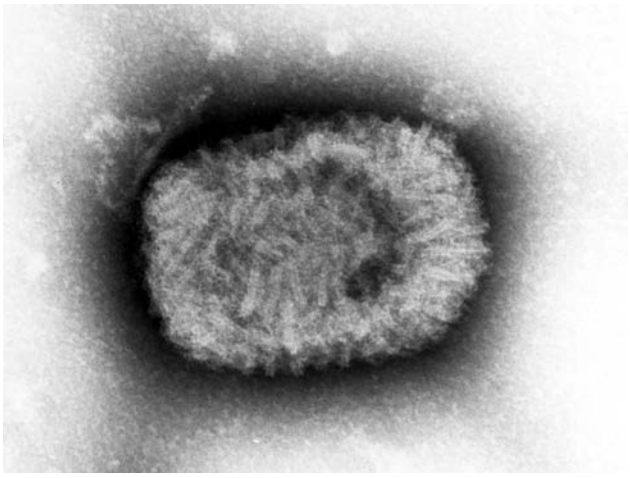


Figure 24-1. A transmission electron micrograph of a tissue section containing variola viruses.
Photograph: Courtesy of FA Murphy, University of Texas Medical Branch, Galveston, Texas.

of a lipoprotein layer embedded with surface tubules enclosing a core described as biconcave. The core contains the viral DNA and core fibrils, and it is surrounded by the core envelope and a tightly arranged layer of rod-shaped structures known as the palisade layer.[19] Between the palisade layer and the outer envelope are two oval masses known as the lateral bodies.

Two forms of orthopoxviruses result from the replication cycle: intracellular mature virion (IMV) and enveloped virion (EV). EV can be further categorized as intracellular enveloped virion (IEV), cell-associated enveloped virion (CEV), and extracellular enveloped virion (EEV). IMV, CEV, and EEV are fully infectious, whereas the ability of IEV to promote productive infection in a naïve cell has not been demonstrated.[18,19] These virus forms will be discussed in greater detail in a later section.

Entry

Entry of orthopoxviruses into permissive cells has been extensively studied and, depending on the methodology used, these studies have produced conflicting results. Electron microscopy has suggested that the attachment of enveloped virions (EEV and CEV) to the cell surface results in the disruption of the envelope and exposes the IMV particle that subsequently binds to the cell surface. In this model, the outer membrane of the IMV particle fuses with the plasma membrane of the cell, releasing the viral core into the cell.[20]

Endocytosis has been suggested as an alternative model of orthopoxvirus entry using video microscopy of fluorescently labeled virus particles. IMV and enveloped virions (CEV and EEV) bind to the cell surface and, following a complex series of signaling events, are internalized by endocytosis. For enveloped particles, it is predicted that low pH and/or exposure to glycosaminoglycans results in the disruption of the envelope and exposure of the IMV particle. The outer membrane of the IMV then fuses with the endosomal membrane, releasing the viral core into the cell cytoplasm.[21]

It is likely that both suggested mechanisms of entry are used by orthopoxviruses and that the method used is dependent on the virus strain and the target cell type. Regardless of how the virus enters a given cell, the final step in entry is the initiation of early gene transcription that is followed by core uncoating and replication.

TABLE 24-1

POXVIRUSES THAT CAUSE HUMAN DISEASE

Genus	Species	Animal Reservoir
Orthopoxvirus	Variola virus	None
	Vaccinia virus	Unknown (none?)
	Cowpox virus	Rodents
	Monkeypox virus	Rodents
Parapoxvirus	Bovine popular stomatitis virus	Cattle
	Orf virus	Sheep
	Pseudocowpox virus	Cattle
	Seal parapoxvirus	Seals
Parapoxvirus	Tanapox	Rodents (?)
	Yabapox virus	Monkeys (?)
Molluscipoxvirus	Molluscum contagiosum virus	None

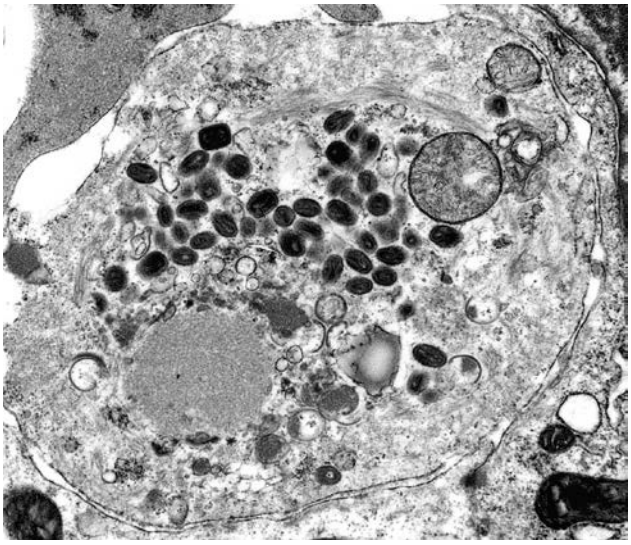


Figure 24-2. Thin section of smallpox virus growing in the cytoplasm of an infected chick embryo cell. Intracellular mature virions (brick-shaped) and immature virions (spherical) are visible. Magnification is approximately × 25,000. Photograph: Courtesy of FA Murphy, University of Texas Medical Branch, Galveston, Texas.

Replication

Orthopoxvirus genomes are linear, double-stranded DNA approximately 200 kb long. The genomes encode between 176 and 266 proteins, including enzymes and factors that are necessary for self-replication and maturation. The central region of the genome contains highly conserved genes that are essential for viral replication, while the terminal regions contain less conserved genes that are important for virus–host interactions. The virus contains a number of virus-encoded enzymes, in particular, a DNA-dependent RNA polymerase that transcribes the viral genome. Early transcripts encode proteins involved in the modulation of the host immune response as well as the DNA polymerase and other enzymes that promote the replication of the viral genome. Uncoating of the core exposes viral DNA and replication occurs in cytoplasmic factories referred to as B-type inclusions, in which virions at various stages of assembly are seen. Whether host cell nuclear factors are involved in viral replication or maturation is unclear. Cells infected with some poxviruses (eg, cowpox, avian poxviruses) also contain electron-dense A-type inclusions, usually containing mature virions; A-type inclusions are easily seen by light microscopy (Figure 24-3).[18,19,21,22]

Nascent viral genomes are used as the templates for the production of intermediate transcripts that encode factors that promote the production of late transcripts. The late transcripts are translated into structural and nonstructural proteins of the virions.[19]

Morphogenesis and Egress

Late (structural and nonstructural) proteins, along with DNA concatemers that are formed during the early phase of replication, are assembled into genomic DNA and packaged into immature virions, which then evolve into the brick-shaped IMV. IMVs, which represent the majority of virus particles produced during infection, are fully infectious; however, their release from the cell is dependent on cell lysis. A small subset of IMV particles gains two additional membranes at either the trans-Golgi network or early endosomes to become IEVs. IEVs migrate to the cell surface via microtubules where their outermost membrane fuses with the cell membrane to form CEVs. CEVs induce the polymerization of actin to form filaments that promote the direct transfer of CEVs to adjacent cells. If CEVs become dissociated from the cell membranes, they become EEVs. Although IMVs are produced in greatest abundance in cell culture and are the most stable to environmental degradation, CEVs and EEVs probably play a more critical role in cell-to-cell spread in the intact animal.[22]

Many of the *Orthopoxvirus* gene products, known as virokines and viroceptors, interact with and modulate essential functions of host cells and immune processses.[18,23] Furthermore, the genomes of many poxvirus species also encode intracellular immune modulators, thus providing a broad and complex mechanism through which these viruses can subvert host immune responses that would be deleterious for infection. Interestingly, cowpox virus, which predominantly causes localized skin lesions in healthy humans, possesses the largest genome (~220 kbp) of the orthopoxvirus species and thus may contain the greatest number of immunomodulatory components to escape host immunity. In light of this, it is unsurprising that cowpox virus is also believed to have the broadest host range species of the orthopoxviruses.[24] The limited host range of variola may relate to the unique association of viral gene products with various host signaling pathways. Therefore, strategies that block such key pathways in the replication and maturation of poxviruses provide potential targets for therapeutic intervention.[19]

Significant efforts have been made to dissect the molecular mechanisms that orthopoxviruses use to modulate host cell signaling networks.[25–27] Investigations such as these provide important information regarding viral pathogenesis and may provide the identities of therapeutic targets for the development of novel orthopoxvirus specific antivirals.

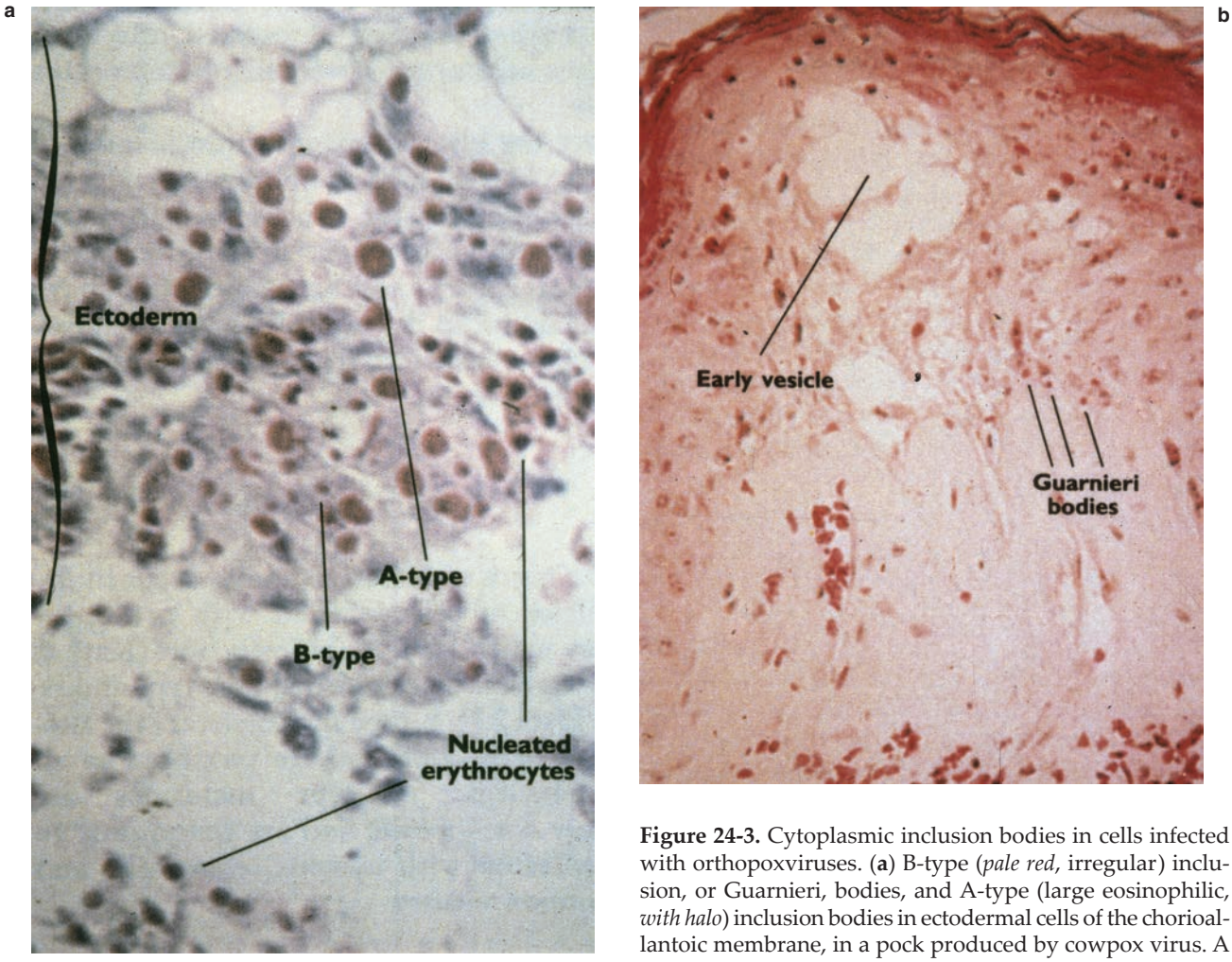


Figure 24-3. Cytoplasmic inclusion bodies in cells infected with orthopoxviruses. (**a**) B-type (*pale red*, irregular) inclusion, or Guarnieri, bodies, and A-type (large eosinophilic, *with halo*) inclusion bodies in ectodermal cells of the chorioallantoic membrane, in a pock produced by cowpox virus. A number of nucleated erythrocytes are in the ectoderm and free in the mesoderm, and the surface of the pock is ulcerated. Hematoxylin-eosin stain. (**b**) This section of the skin of a patient with hemorrhagic-type smallpox shows Guarnieri bodies and free erythrocytes below an early vesicle. Hematoxylin-eosin stain. Reproduced with permission from Fenner F, Henderson DA, Arita I, Jezek Z, Ladnyi ID. *Smallpox and Its Eradication*. Geneva, Switzerland: World Health Organization; 1988: 85.

Phylogenetic Relationships

The evolutionary relationships within the *Orthopoxvirus* genus have been facilitated by the recent availability of complete DNA sequences for more than 30 species. Phylogenetic analysis (Figure 24-4) reveals that at the sequence level, any two virus species contain at least 96% homogeny, variola is more closely related to camelpox and taterapox (isolated from rodents) viruses than any other members of the genus, and vaccinia is most closely related to cowpox virus strain GRI-90.[28–31] Cowpox virus strain GRI-90 appears to be less closely related to cowpox virus strain Brighton, indicating that at least two separate species are included under the name cowpox virus. Monkeypox virus does not group closely with any other orthopoxvirus, which indicates that it diverged from the rest of the genus members long ago. Virulence or attenuation may hinge on a few genetic determinants. For example, variola major (associated with a 30% fatality rate) and variola minor (<1% fatality rate) are greater than 98% identical over the length of the 185,000-kilobase (kb) genome.

When a phylogeny is developed based on gene content instead of genetic sequence, the clustering is slightly different. In the gene content phylogeny, all cowpox virus strains belonging to the same clade are distinctly divergent from vaccinia virus, and camelpox virus is in the same clade as monkeypox virus instead of variola virus.[30] Variola virus, monkeypox virus, and camelpox virus can all be traced back to a common branching point that is divergent from the less pathogenic orthopoxviruses vaccinia virus and

cowpox virus. The discrepancies that exist between the sequence-based and gene content-based phylogenies can predominantly be traced to the variable terminal ends of the genome.[30] Most of the proteins encoded at the terminal ends are known to be involved in virus:host interaction (immunomodulation, virulence, host range restriction).[19] Variola virus, which is strictly a human pathogen, has the fewest genes at its terminal ends whereas cowpox virus, which is capable of causing disease in numerous mammalian species, has the greatest number of genes at its terminal ends. It has been postulated that, through genetic reduction, variola virus has maintained only the genes essential to productive infection in humans. By minimizing the number of genes with potential overlapping or antagonistic functions, it is thought that variola virus became a highly adapted and efficient pathogen. In contrast, cowpox virus is capable of infecting a wide variety of mammalian species (including humans) but is not highly pathogenic in any of those species. The large number of genes at its terminal ends improves the host range of cowpox virus but decreases its overall efficiency. Regardless of phylogeny, all orthopoxviruses are antigenically similar allowing for cross protection.[30]

Viral neutralizing epitopes are associated with structural proteins encoded by genes located in the central conserved region of the viral genome, and they are present on the surface of IMVs as well as on the envelope of EEVs and CEVs.[32–34] Given the conserved nature of these proteins, epitopes are relatively uniform for all members of the genus *Orthopoxvirus*. Cross protection allowed for the development of the vaccine that eradicated smallpox, and it continues to be a fundamental key to the development of vaccinia based countermeasures against orthopoxviruses.[35–37]

Pathogenesis

Most knowledge about smallpox pathogenesis is inferred from animal studies of vaccinia in mice, mousepox, rabbitpox, monkeypox, and from vaccinia in humans.[16,38–40] Studies using nonhuman primates infected with variola corroborate these findings and lend further insight into human smallpox and monkeypox infections; however, certain deficiencies exist with the model.[41] The production of a clinical syndrome in cynomolgus macaques that resembles human smallpox requires intravenous infection (an unnatural route)

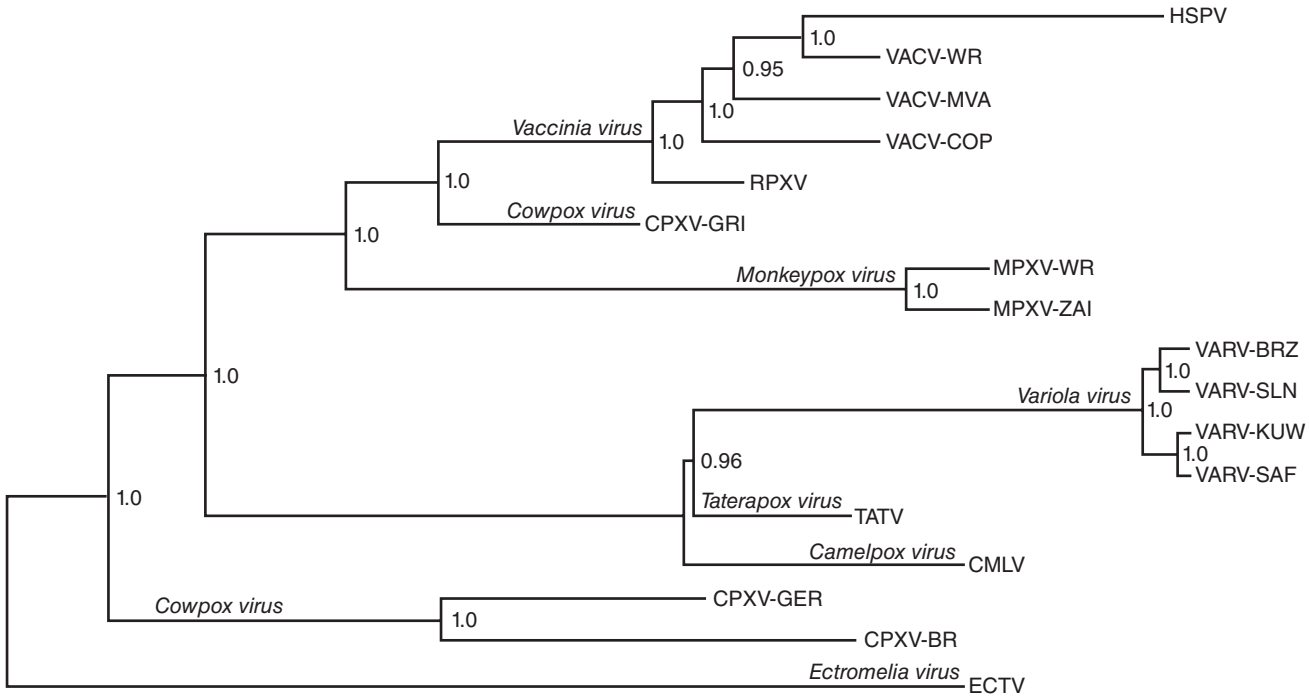


Figure 24-4. Orthopoxvirus gene sequence phylogenetic tree. Species names are noted on the branch line that separates different clades. Specific virus strains are indicated at the terminal point of the line. The numerical values represent the confidence predication using Bayesian inference for each clade. Reproduced (in accordance with the creative commons attribution license associated with open access articles [http://creativecommons.org/licenses/by/3.0]) from Hendrickson RC, Wang C, Hatcher EL, Lefkowitz EJ. Orthopoxvirus genome evolution: the role of gene loss. *Viruses*. 2010;2:1933–1967.

with an extremely high dose of inoculum; and even then, mortality in this model is inconsistent.[41] In both natural and experimental aerosol infections, the virus is introduced via the respiratory tract, where it first seeds the mucous membranes. Although it is unclear whether alveolar macrophages are responsible for the transport of virus, the first round of replication occurs in the lung draining lymph nodes, followed by a transient viremia, which seeds tissues, especially those of the reticuloendothelial system, including regional lymphatics, spleen, and tonsils.[42,43] A second, brief viremia transports the virus to the skin and visceral tissues immediately before the prodromal phase. In humans, the prodrome is characterized by an abrupt onset of headache, backache, fever, and a sore throat as a result from viral replication in the oral mucosa. Characteristic skin lesions develop with a centrifugal distribution and synchronous progression following viral invasion of the capillary epithelium of the dermal layer. The virus may also be present in urine and conjunctival secretions.[43] At death, most visceral tissues contain massive virus concentrations.

In a review of all pathology reports published in English over the past 200 years,[44] Martin suggested that generally healthy patients who died of smallpox usually died of renal failure, shock secondary to volume depletion, and difficulty with oxygenation and ventilation as a result of viral pneumonia and airway compromise, respectively. Degeneration of hepatocytes may have caused a degree of compromise, but liver failure was not usually the proximate cause of death.

Much of the human pathogenesis of smallpox remains a mystery because of the limited tools that were available when it was an endemic disease. Comparisons between the limited clinical and pathological data from human smallpox victims with the pathophysiology of disease from monkeypox and variola nonhuman primate infection models suggest a role for the dysregulation of immune responses responsible for the production of proinflammatory cytokines, lymphocyte apoptosis, and the development of coagulation abnormalities. High viral burdens, which were identified in numerous target tissues in the animal models, were likely associated with organ dysfunction and multisystem failure. Immunohistochemistry staining confirmed the distribution of viral antigen, while electron microscopy demonstrated evidence of replicating virus, which correlated with pathology observed in the lymphoid tissues, skin, oral mucosa, gastrointestinal tract, reproductive system, and liver. Apoptosis, particularly within the T-cell population, was a prominent observation in lymphoid tissues, although the cause of this widespread apoptosis remains unknown. However, the strong production of proinflammatory cytokines is due at least in part to the upregulation of various proapoptotic genes. The strong upregulation of cytokines may have also contributed to the development of a hemorrhagic diathesis. The detection of D-dimers and other changes in hematologic parameters in monkeys that developed classical or hemorrhagic smallpox suggests that activation of the coagulation cascade is a component of both disease syndromes. However, in human populations, the occurrence of hemorrhagic smallpox was approximately 1% to 3% of the total cases observed.[2,23,39,44,45]

From these recent studies of variola and monkeypox virus infection in nonhuman primates, the "toxemia" described by clinicians for human smallpox may be fundamentally related to the processes underlying septic shock.[2,46] Common denominators include lymphocyte apoptosis; proinflammatory cytokines (exuberant production of type I interferon [IFN], interleukin-6, tumor necrosis factor-α, and IFN-γ measurable in plasma); and disseminated intravascular coagulation. Aberrant activation of these pathways, which contributes to toxic shock, is a hallmark of pathological activation of the innate immune system.

To facilitate viral replication, orthopoxviruses generally modulate their host's immune response to the pathogen's advantage. Poxviruses encode proteins that target or interrupt the natural inflammatory response and interfere with apoptosis, synthesis of steroids, and initiation of the complement system. In general, these proteins block either extracellular immune signals (by mimicking or interfering with cytokine/chemokine proteins and/or receptors), or they work intracellularly by interfering with apoptosis, targeting by the immune system, or intracellular immune cell signaling. A combination of these mechanisms may allow the virus to overcome immunological surveillance and establish clinical disease in the host.[47]

ORTHOPOXVIRUSES AS BIOLOGICAL WARFARE AND BIOTERRORISM THREATS

Using variola virus in warfare is an old concept. British colonial commanders used blankets from smallpox victims as a biological weapon, distributing them among Native Americans.[48–50] During the American Civil War, allegations were made about the use of variola as a biological weapon, although no definite evidence existed.[51,52] In the years leading up to and during World War II, the Japanese military explored

weaponization of variola during the operations of Unit 731 in Mongolia and China. More recently, the former Soviet Union developed smallpox as a strategic weapon and produced ton quantities of liquid variola virus on a continuing basis well into the 1980s.[10,53] The former Soviet Union also conducted open air testing of weaponized variola and demonstrated that infectious virus could infect humans 15 km downwind.[7]

Although declared stocks of variola virus exist only at the two WHO repositories (the Centers for Disease Control and Prevention [CDC] in Atlanta, Georgia, USA, and at the State Research Center of Virology and Biotechnology Vector in Koltsovo, Novosibirsk Oblast, Russia), it is of concern that undeclared stocks may exist in military sites within the former Soviet Union, or that they were transferred from the Soviet program to programs in Iraq, Iran, North Korea, or elsewhere.[53] The probability that such stocks exists is impossible to assess, but the catastrophic consequences of smallpox release in a biological attack cannot be discounted.[4]

Variola is a significant threat for use as a biological weapon because of its stability, infectivity in aerosol form, small infectious dose, severe disease manifestations, and interhuman transmissibility. Furthermore, the anticipated morbidity and mortality for the general population may be higher than historical averages resulting from waning immunity following vaccinations in the distant past and immunosuppression as a result of HIV, cancer, organ transplants, and old age.[3] Other members of the *Orthopoxvirus* genus share many of variola's properties and are potential agents of a deliberate bioterrorist attack. Of the poxviruses other than variola, monkeypox virus presents the greatest threat for biological warfare or terrorism use. Monkeypox can naturally produce severe disease in humans that closely resembles smallpox, with mortality exceeding 15% in some outbreaks.[54] The disease can be transmitted from person to person, is highly transmissible by aerosol and, in at least some nonhuman primate models, has an infectious dose as low as one tissue culture infecting dose ($TCID_{50}$).[39,55–57] Monkeypox virus, like variola, is relatively stable and can resist desiccation in both heat and cold.[58] Monkeypox virus also grows to high titers in cell culture systems, including the chick chorioallantoic membrane of embryonated eggs, a simple methodology described in older microbiology texts using equipment and supplies available at agricultural supply stores. A large dose of monkeypox delivered by aerosol can produce a rapidly progressive and overwhelming pneumonia in nonhuman primate models.[40] Furthermore, monkeypox virus may have already been weaponized by the former Soviet military.[10]

Cowpox and buffalopox produce limited cutaneous disease in humans in natural infection.[17] Buffalopox, like cattlepox, may be essentially identical to vaccinia.[59] The effect of altering route of delivery, dose of virus, or the actual viral agent itself on human disease manifestation is unclear. Several studies demonstrate that orthopoxviruses produce different clinical syndromes and immunological responses in animal models depending on the route of infection.[40,60–64] Aerosol infection has the potential to produce more pronounced pulmonary disease.[40,56,65] In addition, all orthopoxviruses share a significant amount of homology with variola and monkeypox.[29] If the critical virulence factors for systemic human disease were determined, then cowpox, buffalopox, or other orthopoxviruses could potentially be genetically modified to express these critical factors. When designed as a weapon and delivered by aerosol, these viruses could have a significant impact in humans, even without genetic modification.

Camelpox rarely, if ever, causes disease in humans. However, because of Iraqi admissions of research with camelpox as part of the country's biological warfare program, some concern exists over its potential use as a biological weapon.[66] Camelpox virus is a close relative of variola virus; the major difference between camelpox virus and variola major virus strain Bangladesh-1975 genomes is four additional insertions, elongated inverted terminal repeats, and a small area of gene rearrangement present in camelpox virus.[28] As with other orthopoxviruses, slight modifications in the camelpox virus genome may dramatically change its pathogenicity in humans. Although prohibited by US law, genetic modification of camelpox would be a likely starting point by any group that wanted to construct variola based on published sequences. In addition, it is now technically feasible to create infectious variola using an oligonucleotide synthesizer, analogous to the recent demonstration for creation of the much simpler polio virus.[31,67,68]

The possibility of genetically engineered orthopoxviruses remains unknown in biodefense research. Studies have shown increased mousepox and vaccinia virus virulence in mouse models by the incorporation of cloned host cytokine genes into the virus genome.[69,70] Whether these results represent findings unique to the virus–host model used or reflect a more general premise of enhanced virulence is unclear.[71,72] The possibility of similar genetic engineering only increases the threat of orthopoxviruses that are not significant natural threats for human disease. Further research is warranted to ensure that present and future countermeasures are effective with modified viruses.

CLINICAL ASPECTS OF ORTHOPOXVIRUS INFECTIONS

Smallpox

Variola virus is stable and retains its infectivity for long periods outside the host.[73] Variola virus is infectious by aerosol, but natural airborne spread other than among close contacts is unusual.[3,74,75] Approximately 30% of susceptible contacts became infected during the era of endemic smallpox,[76] and the WHO eradication campaign was predicated upon the requirement of close person-to-person proximity for reliable transmission to occur. Nevertheless, two hospital outbreaks demonstrated that variola virus can be spread through airborne dissemination in conditions of low relative humidity.[77] The patients in these outbreaks were infectious from the onset of their eruptive exanthem, most commonly from days 3 through 6 after fever onset. If the patient had a cough, then chances of virus transmission were greatly increased. Indirect transmission via contaminated bedding or other fomites was infrequent.[78] Some people in close contact with patients harbored virus in their throats without developing disease and may have been a means of secondary transmission.[43,79]

After exposure to aerosolized virus, variola travels from the upper or the lower respiratory tract to regional lymph nodes, where it replicates and gives rise to a primary viremia.[2] The incubation period of smallpox averages 12 days (range 9–14 days). Those in contact with infected patients were quarantined for a minimum of 16 to 17 days following exposure to ensure an adequate window of time had passed without the appearance of symptoms.[2] During normal smallpox disease, variola virus was sporadically recovered from the blood, but not nearly at the levels detected in patients with hemorrhagic smallpox.[80] After replication in regional lymph nodes virus disseminated systemically to other lymphoid tissues, spleen, liver, bone marrow, and the lungs, and created a secondary viremia. Clinical manifestations began acutely with malaise, fever, rigors, vomiting, headache, and backache; 15% of patients developed delirium. Approximately 10% of light-skinned patients exhibited an observable erythematous rash during this phase. After 2 to 3 more days, an exanthem appeared concomitantly with a discrete rash about the face, hands, and forearms. Given the lack of a keratin layer on mucous membranes, lesions shed infected epithelial cells and give rise to infectious oropharyngeal secretions in the first few days of the eruptive illness, and occasionally 24 hours before eruption.[81] These respiratory secretions were the most significant but not the sole means of virus transmission. Following subsequent eruptions on the lower extremities, the rash spreads centrally to the trunk. Lesions quickly progressed from macules to papules and eventually to pustular (umbilicated) vesicles, and were more abundant on the extremities and face (Figure 24-5). This centrifugal distribution of lesions is an important diagnostic feature, typical of smallpox disease. In contrast to the lesions seen in varicella (chickenpox), smallpox lesions on various segments of the body remain generally synchronous in their stage of development. From 8 to 14 days after onset, the pustules form scabs, which leave depressed depigmented scars upon healing. Although variola titers in the throat, conjunctiva, and urine diminish with time, virus can readily be recovered from scabs throughout convalescence.[2,82] Therefore, patients should be isolated and considered infectious until all scabs separate.

Two distinct forms of smallpox were recognized in the last century of smallpox occurrence: variola major and variola minor. Variola major, the highly virulent, prototypical, and historically significant form of the disease, remained prevalent in Asia and parts of Africa during the 20th century. Analysis conducted on human viral isolates indicated a distinct evolution of three virus clades (A, B, C) associated with geographical distribution and case fatality rates.[31]

Although isolates from clade C, found predominantly in Asia, were associated with an overall higher case fatality rate compared to A and B, virus evolution was present, as evidenced by the attenuated strains detected in Africa. Variola minor was distinguished by milder systemic toxicity and more diminutive pox lesions.[2] However, Dixon reported many cases that were indistinguishable from variola major in his extensive comparison of lesion types.[83] Korte first described variola minor, found in Africa, in 1904.[2] Chapin found a similar mild form known as alastrim that occurred in North America as early as 1896 and subsequently was exported to South America, Europe, and Australia.[84] Two distinct viral strains of reduced virulence caused variola minor and alastrim, and both typically caused 1% mortality in unvaccinated victims.[2]

The Rao classification specified five clinical presentations of variola.[85] Three-quarters of variola major cases were designated classic or ordinary type (Figure 24-5). After prodromal fever and constitutional symptoms appeared, patients developed the typical variola rash, centrifugal in distribution, with synchronous progression from macules to papules, to vesicles to pustules, and then to scabs. The fatality rate was 3% in vaccinated and 30% in unvaccinated patients.



Figure 24-5. This series of photographs illustrates the evolution of skin lesions in an unvaccinated infant with the classic form of variola major. (**a**) The third day of rash shows synchronous eruption of skin lesions; some are becoming vesiculated. (**b**) On the fifth day of rash, almost all papules are vesicular or pustular. (**c**) On the seventh day of rash, many lesions are umbilicated, and all lesions are in the same general stage of development. Reproduced with permission from Fenner F, Henderson DA, Arita I, Jezek Z, Ladnyi ID. *Smallpox and Its Eradication*. Geneva, Switzerland: World Health Organization; 1988: 10–14. Photographs by I Arita.

Other clinical presentations of smallpox occurred less frequently, probably because of the difference in host immune response. Flat-type smallpox, noted in 2% to 5% of smallpox patients, was characterized by both severe systemic toxicity and the slow evolution of flat, soft, and focal skin lesions that did not resemble the classical variola exanthem (Figure 24-6). This syndrome caused 66% mortality in vaccinated patients and 95% mortality in unvaccinated patients.[85] Fewer than 3% of smallpox patients developed hemorrhagic-type smallpox, which was accompanied by extensive petechiae (Figure 24-7), mucosal hemorrhage, and intense toxemia; death usually occurred before typical pox lesions developed.[86] However, on occasion hemorrhagic smallpox also occurred as a late stage complication of classical smallpox disease. Both hemorrhagic-type and flat-type smallpox may have indicated underlying immunodeficiency; hemorrhagic forms occurred more commonly in pregnant women and young children.[87] The modified type, which occurred typically (but not exclusively) in previously vaccinated individuals, was characterized by moderation of constitutional symptoms and typically reduced numbers of and a rapid evolution of lesions, with scabs formed by the 9th day of the illness. The *variola sine eruptione* was characterized by prodromal fever and constitutional symptoms. These patients, most of whom had been vaccinated, never developed a rash.[85] In actuality, the manifestations of variola infection fell along a spectrum, and classification was primarily for the purpose of prognosis.

Bacterial superinfection of pox lesions was relatively common in the preantibiotic era, especially in the absence of proper hygiene and medical care in tropical environments.[2] Arthritis and osteomyelitis developed late in the disease in about 1% to 2% of patients, occurred more frequently in children, and often manifested as bilateral joint involvement, particularly of the elbows.[88] Viral inclusion bodies could be demonstrated in the joint effusion and bone marrow of the involved extremity. Cough and bronchitis were occasionally reported as prominent manifestations of smallpox, with implications for spread of contagion; however, pneumonia was unusual.[2] Pulmonary edema occurred frequently in hemorrhagic-type and flat-type smallpox. Orchitis was noted in approximately 0.1% of patients. Encephalitis developed in 1 in 500 cases of variola major, compared with 1 in 2,000 cases of variola minor. Keratitis and corneal ulcers were important complications of smallpox, progressing to blindness in slightly less than 1% of cases. Disease during pregnancy precipitated high perinatal mortality, and congenital infection was also recognized.[2]

Partial immunity through vaccination resulted in modified-type smallpox, in which sparse skin lesions evolved variably, often without pustules, and quickly, with crusting occurring as early as the 7th day of illness. When exposed to smallpox, some fully immune individuals developed fever, sore throat, and conjunctivitis (called contact fever), which lasted several days but did not give rise to the toxicity or minor skin lesions that signify *variola sine eruptione*. Persons who recovered from smallpox possessed long-lasting immunity, although a second attack may have occurred in 1 in 1,000 persons after an intervening period of 15 to 20 years.[89] Both humoral and cellular responses are important components of recovery from infection. Neutralizing antibodies peak 2 to 3 weeks following onset and last longer than 5 years and up to several decades in some individuals.[34,45,90]



Figure 24-6. Flat-type smallpox in an unvaccinated woman on the sixth day of rash. Extensive flat lesions (**a** and **b**) and systemic toxicity with fatal outcome were typical. Reproduced with permission from Fenner F, Henderson DA, Arita I, Jezek Z, Ladnyi ID. *Smallpox and Its Eradication*. Geneva, Switzerland: World Health Organization; 1988: 33. Photographs by F Dekking.

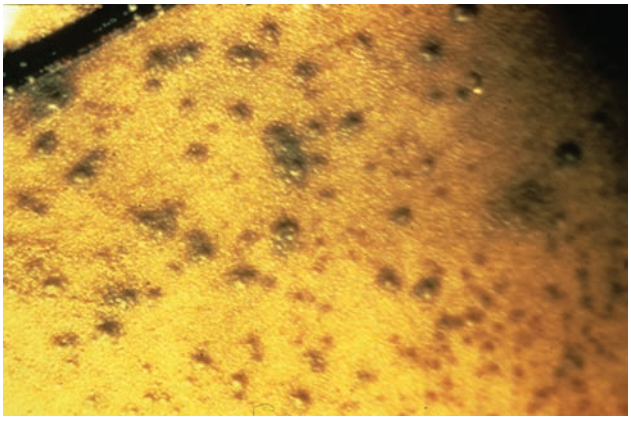


Figure 24-7. Early hemorrhagic-type smallpox with cutaneous signs of hemorrhagic diathesis. Death usually occurred before the complete evolution of pox lesions. Reproduced with permission from Herrlich A, Munz E, Rodenwaldt E. *Die pocken; Erreger, Epidemiologie und klinisches Bild*. 2nd ed. Stuttgart, Germany: Thieme; 1967. In: Fenner F, Henderson DA, Arita I, Jezek Z, Ladnyi ID. *Smallpox and Its Eradication*. Geneva, Switzerland: World Health Organization; 1988: 35.

Monkeypox

The clinical features of human monkeypox are classically described as being similar to those of smallpox.[91] Disease begins with a 2- to 4-day disruptive phase with high fever and prostration. The rash develops and progresses synchronously over 2 to 4 weeks, evolving from macules to papules, to vesicles and pustules, to scabs. Lesions are usually umbilicated, have a centrifugal distribution, and involve the palms and soles. Sore throat and frank tonsillitis frequently occur during the eruptive phase of human monkeypox.[91,92] Lymphadenopathy is a common finding that differentiates monkeypox from smallpox. This has been documented in up to 83% of unvaccinated persons with monkeypox and arises most frequently early in the course of infection, involving the submandibular and cervical nodes and less frequently the axillary and inguinal nodes.

Clinical manifestations of human monkeypox are likely more diverse, and not as stereotypical as those of smallpox. Mild infections were frequent in the first recognized African cases, with 14% of patients having fewer than 25 lesions and no incapacity.[91] In a series of 282 patients (of which 250 were not vaccinated), the exanthema first appeared somewhere other than the face in 18% of the unvaccinated patients; 31% of vaccinated patients had pleomorphic or "cropping" appearance of rash lesions, and 9.4% had centripetal distribution.[93] All of these features were inconsistent with a mimic of smallpox. Patients in the 2003 US outbreak tended to have fewer mild lesions than most African patients. Patients were hospitalized in only 19 of 78 suspected cases in the United States, and only 2 had significant illness requiring some form of medical intervention.[94,95] A surveillance study conducted from 2005 to 2007 in the DRC laboratory confirmed 760 cases, which suggests a 20-fold increase in human monkeypox incidence since the 1980s in the same health zone.[13]

Monkeypox virus can be subdivided into two distinct clades that are genetically, clinically, and geographically distinct. The Congo Basin monkeypox virus clade has associated case fatality rates of approximately 10% in nonvaccinated individuals,[96] as opposed to the reduced pathogenicity and transmissibility of the West African clade of monkeypox (which caused the US outbreak).[57,97] Comparative infection models in nonhuman primates, mice, prairie dogs, and ground squirrels have all demonstrated greater lethality or morbidity associated with Congo Basin monkeypox virus infection as compared to West African monkeypox virus.[98–101] A *sine eruptione* form of monkeypox has not been described, but the number of serologically diagnosed infections without consistent rash illness suggests it is a possibility.[102] A hemorrhagic form of human monkeypox has not been documented.[103,104]

Complications of monkeypox are more common in unvaccinated persons and children.[96] During intensive surveillance in the DRC between 1980 and 1986, secondary bacterial superinfection of the skin was the most common complication (19.2% of unvaccinated patients), followed by pulmonary distress/pneumonia (11.6% of unvaccinated patients), vomiting/diarrhea/dehydration (6.8% of unvaccinated patients), and keratitis (4.4% of unvaccinated patients). With the exception of keratitis, the incidence of these complications in vaccinated persons was at least 3-fold less. Alopecia has been noted in some cases.[105] Encephalitis was detected in at least one monkeypox case in the DRC and in one of the cases in the US outbreak of 2003.[93,95] As in smallpox, permanent pitted scars are often left after scabs separate.

Severity of disease and death is related to age and vaccination status, with younger unvaccinated children faring worse.[91,105–107] The case fatality rate in Africa varied in different outbreaks and periods of increased surveillance. The fatality rate was 17% from 1970 through 1979, 10% from 1981 through 1986, and 1.5% from 1996 through 1997.[54] The low fatality rates in certain outbreaks were influenced by the lack of proper laboratory confirmation to exclude cases of varicella virus from monkeypox virus infections. It is believed no fatalities occurred among the 78 suspected

cases in the 2003 US monkeypox outbreak, at least in part because the less virulent West African strain was responsible.[94] The presence of comorbid illnesses, such as measles, malaria, or diarrheal disease, may have a significant impact on mortality in children.[96] Cause of death in monkeypox is not universally clear, although 19 of 33 fatalities in one series of patients involved pulmonary distress or bronchopneumonia, suggesting superimposed bacterial pneumonia.

Other Orthopoxviruses Infecting Humans

Cowpox is primarily a localized, cutaneous disease.[17] Baxby, Bennett, and Getty reviewed 54 cases of cowpox infection with a detailed discussion of clinical manifestations.[108] Disease usually consists of single pock-like lesions on the hands or face, although multiple lesions are seen in roughly one-quarter of cases. Typical lesions progress from macule to papule to vesicle to pustule to dark eschar, with a hemorrhagic base being common in the late vesicular stage. Progression from macule to eschar is slow, often evolving over 2 to 3 weeks. Local edema, induration, and inflammation are common and can be pronounced. Lesions are painful and are accompanied by regional lymphadenopathy. Complete healing and scab separation usually occur within 6 to 8 weeks of onset, but may take 12 weeks or longer. A majority of patients experience some constitutional symptoms before the eschar stage.

The majority of human cowpox infections are self-limited and without complication. Ocular involvement, including the cornea, can occur, but it usually resolves without permanent damage. A few severe generalized cowpox infections have been reported including one fatality.[108,109] Three of these four described cases included a history of atopic dermatitis, indicating a risk of increased severity of disease analogous to vaccinia.

Buffalopox and cattlepox infections in humans have not been extensively described but have been observed in areas of Brazil. Limited data suggest human infection usually occurs on the hands and consists of inflamed and painful pustular lesions progressing through a Jennerian evolution.[110–112] Regional lymphadenopathy and fever can accompany local disease.[112]

DIAGNOSIS

Clinical Diagnosis

The clinical presentation of smallpox is similar to many vesicular and pustular rash illnesses, including varicella, herpes simplex, drug reactions, and erythema multiforme. Although the index of suspicion for an eradicated disease may be low, the failure to recognize a case of smallpox could result in the exposure of hospital contacts and the seeding of an outbreak. The smallpox diagnosis and evaluation page on the CDC website (http://www.bt.cdc.gov/agent/smallpox/diagnosis/) is an essential resource to assist a clinician in evaluating a febrile patient presenting with a rash. This site contains an algorithm to quickly determine the likelihood of clinical smallpox and a standardized worksheet to classify the risk of smallpox using the CDC criteria.

Specimen Collection and Handling

Collection of appropriate specimens is paramount for accurate laboratory diagnosis of orthopoxvirus infection. Ideally, cutaneous tissue (from skin lesions) and blood are sent for diagnostic testing, with other samples being sent at the request of public health officials or experts in the field.[104] Detailed instructions for specimen collection can be found in the Department of Defense Smallpox Response Plan (http://www.bt.cdc.gov/agent/smallpox/responseplan/index.asp) or on the CDC website (http://www.cdc.gov/ncidod/monkeypox/index.htm). Briefly, vesicles or pustules should be unroofed, the detached vesicle skin sent in a dry tube, and the base of the lesion scraped to make a touch-prep on a glass slide. Biopsy specimens should be split (if possible) and sent in formalin and in a dry tube. If scabs are collected, two scabs should be sent in a dry tube. Dacron or polyester swabs should be used for oropharyngeal swabs and transported in dry tubes. Blood should be collected in a serum separator tube (which is then centrifuged to separate serum) and in an anticoagulant tube for whole blood. Clinical specimens potentially containing orthopoxviruses other than variola virus, including monkeypox virus, may be handled in a biosafety level 2 using biosafety level 3 practices (specimens potentially containing variola virus must be handled in biosafety level 4).[113]

Many phenotypic and genotypic methods involving virological, immunological, and molecular approaches have been used to identify orthopoxviruses.

Phenotypic Diagnosis

In the past, a presumptive diagnosis of orthopoxviruses required a laboratory with capabilities and expertise in viral diagnostics. Microscopists with experience in poxvirus infections can often recognize

the characteristic inclusion bodies (Guarnieri bodies, corresponding to B-type poxvirus inclusions [see Figure 24-3]) in tissue samples under light microscopy. These cytoplasmic inclusions are hematoxylinophilic, stain reddish purple with Giemsa stain, and contain Feulgen-positive material.[114] Electron microscopy reveals the unmistakable brick-like morphology of orthopoxviruses in thin sections of infected materials.

Microscopy alone cannot differentiate members of the genus *Orthopoxvirus*, yet the epidemiological setting can suggest which species is involved. The orthopoxviruses with pathogenicity in humans (with the exception of molluscum contagiosum) can be grown on the chorioallantoic membranes of 12-day-old embryonated chicken eggs, where they form characteristic pocks. These viruses also grow readily in easily obtained cell cultures, including VERO cells and additional monkey kidney cell lines, A549 cells, and others. Variola could characteristically be differentiated from other viruses by a strict temperature cut-off at 39°C. Methods for isolation and identification of individual virus species have been reviewed.[115–117] For example, immunogold staining prior to electron microscopy permits a more precise identification to the species level.

Immunodiagnosis

Serologic testing for anti-*Orthopoxvirus* antibodies is an old technique, and various assays were used extensively in the study of smallpox.[2] Current common approaches include enzyme-linked immunosorbent assay (ELISA), plaque reduction neutralization test (PRNT), and immunofluorescence microscopy. Although these assays are proficient at demonstrating the presence of an orthopoxvirus infection, antigenic similarity that results in serologic cross-reactivity makes species differentiation extremely difficult.

ELISAs and PRNTs can be used to detect orthopoxvirus antibodies in a serum sample; however, data interpretation is different. ELISA assays measure the total amount of antibody present in a given serum sample, and they can measure both immunoglobulin M (IgM) and IgG antibodies, allowing for the identification of markers for both active and prior infection. Since IgM antibodies disappear within 6 months, IgM ELISAs can be used to detect recent infections when virus detection is not possible after lesions have healed and scabs have separated. In the investigation of the 2003 US monkeypox outbreak, the CDC relied on anti-*Orthopoxvirus* IgG and IgM ELISAs for serologic diagnosis.[95] More recently, a combination of T-cell measurements and a novel IgG ELISA were used to enhance epidemiological follow-up studies to this outbreak.[118,119] It has also been suggested that, by using linear peptides as antigens, species-specific orthopoxvirus ELISA assays can be developed. Although ELISAs can be sensitive, quick, and easy to perform, they do not provide information as to antibody functionality and their ability to neutralize orthopoxviruses in vitro, or any inferred protective immunity. To determine the neutralizing (ie, inferred protective immunity) antibody titer, a PRNT must be performed. The theoretical protective immunity value for serum antibody levels against variola virus is greater than or equal to 1:40 as determined by PRNT.[120] PRNT cannot differentiate between IgM and IgG antibodies, and orthopoxvirus species-specific PRNT assays have not been developed.

Similar to ELISAs, immunofluorescence microscopy has been used to detect IgM in acute infection directed against cowpox.[109] The technique used is similar to ELISA except that a fluorescent tag attached to the detection antibody allows visual, colorimetric observation of orthopoxvirus antibodies. Immunofluorescence microscopy is not a quantitative assay and only allows determinations of presence versus absence. Quantification can be performed using a fluorescence plate reader. Similar to ELISA, this assay will not provide information on protective antibody levels.

Nucleic Acid Diagnosis

The molecular diagnostic approaches, including DNA sequencing, polymerase chain reaction (PCR), restriction fragment-length polymorphism (RFLP), real-time PCR, and microarrays, are more sensitive and specific than the conventional virological and immunological approaches. Of these techniques, sequencing provides the highest level of specificity for species or strain identification, but current sequencing techniques are not yet as practical as rapid diagnostic tools in most laboratories. RFLP analysis and microarray genotyping also provide high levels of specificity and when combined with PCR, these approaches can offer high levels of sensitivity.[121–123]

Successful performance of PCR-based diagnostics requires extraction of DNA from body fluid and tissue samples, careful design of oligonucleotide primers and probes, and optimization of amplification and detection conditions. Numerous commercial nucleic acid purification methods are available for various sample types, which involve cell lysis and protein denaturation followed by DNA precipitation or fractionation by reversible binding to an affinity matrix. Selection of appropriate primers and probes, and optimization of assay conditions require knowledge of genome sequences and molecular biology techniques.

One of the basic techniques used in PCR-based diagnostics uses PCR-amplified regions of the genome separated on agarose gels by electrophoresis, where the amplicon sizes are used to identify the sample. Several PCR gel-analysis assays have been used to identify cowpox, monkeypox, vaccinia, and variola viruses from clinical specimens.[117,124–126]

Large fragment (LPCR-RFLP) analysis requires amplifying large DNA fragments with high fidelity DNA polymerase enzymes. The amplified LPCR products are purified on agarose gels and digested with a restriction enzyme. The digested DNA fragments are then electrophoresed on polyacrylamide gels for a constant period at constant voltage and stained with ethidium bromide. The restriction pattern is then visualized and photographed with a digital camera. The positions for all DNA fragments in each restriction pattern are determined and digitized by appropriate fingerprinting software. From this pattern, a similarity coefficient is calculated for every pair of restriction patterns and used as an index for species differentiation.

Real-time PCR methods provide exquisite levels of sensitivity and specificity.[127] Real-time PCR is the measurement, by fluorescence detection, of the amount of nucleic acids produced during every cycle of the PCR. Several detection chemistries, such as the intercalating dyes (SYBR Green, Applied Biosystems, Foster City, CA), hydrolysis probes (5′ nuclease or Taqman, Minor Groove Binding Proteins [MGBP]), hybridization probes (Fluorescence Resonance Energy Transfer [FRET]) and molecular beacons, are used. There are several commercially available instruments for real-time PCR, such as the ABI—7900 (Applied Biosystems, Foster City, CA), Smart Cycler (Cepheid, Synntvale, CA), LightCycler (Roche Diagnostics Corporation, Indianapolis, IN), MJ Opticon (Bio-Rad, Hercules, CA), RotorGene (Corbett Life Science, Sydney, Australia), RAPID (Idaho Technology, Salt Lake City, UT), and others. When combined with portable analytical platforms such as the Smart Cycler or LightCycler, real-time PCR systems can be readily deployed to field sites for rapid testing. Real-time PCR assays, which can be performed in a few hours, can test clinical specimens for all orthopoxviruses or for specific species such as vaccinia, variola, or monkeypox.[127–130]

Real-time PCR was one of the diagnostic techniques used in the investigation of the 2003 US monkeypox outbreak.[95] It has also been used during monkeypox active disease surveillance studies in the DRC, identifying 760 new human cases of monkeypox between November 2005 and November 2007.[13] Because of its sensitivity, rapidity, and ease of use, real-time PCR will likely become the primary method of preliminary diagnosis of *Orthopoxvirus* infection, with isolation and growth in a high-level containment laboratory reserved for confirmation.

MEDICAL MANAGEMENT

Prophylaxis

Vaccination

History. Attempts to use infected material to induce immunity to smallpox date to the first millennium; the Chinese used scabs or pus collected from mild smallpox cases to infect recipients usually via insertion of bamboo splinters into the nasal mucosa. This procedure produced disease in a controlled situation that was typically milder than naturally occurring disease and allowed for isolation or controlled exposure of nonimmune individuals. The practice spread to India and from there to Istanbul, where Europeans encountered it in the early 18th century. In Europe, the inoculation of the skin with infected pock material was later referred to as variolation to distinguish the procedure from vaccination. Inducing immunity using variola-contaminated materials had been known to the British Royal Medical Society through Joseph Lister's reports from China as early as 1700, but the procedure was not practiced until Lady Mary Wortley Montagu, wife of the British ambassador to Turkey, introduced it to British society. Lady Montagu, who had been badly disfigured from smallpox, had her son inoculated in Constantinople in 1717 and subsequently arranged for surgeon Charles Maitland to inoculate her daughter in 1722. In the British American colonies, Cotton Mather of Boston persuaded Dr Zabdiel Boylston to conduct variolation on 224 people in 1721 after reading about inoculation in a Royal Medical Society publication.[83] During a smallpox outbreak in Boston in 1752, more than 2,000 persons underwent variolation, resulting in a 90% reduction in mortality among the population immunized. During the Revolutionary War, the Canadian Campaign failed largely because the American reinforcements contracted smallpox. Continued problems with recurring smallpox epidemics among recruits to the Continental Army resulted in a directive in 1779 for variolation of all new recruits. General Washington, who had undergone variolation himself as a young man, was the first military commander to order immunization of his forces.[131]

The practice of variolation, which was never widely accepted, was outlawed at times because many of those inoculated developed grave clinical illness. Variolation

often caused a 1% to 2% mortality rate, and the individuals who died had the potential to transmit natural smallpox. Edward Jenner overcame problems of inoculation with variola by capitalizing on the long-held observation that milkmaids had clear complexions (without smallpox scars), presumably because they had had cowpox, which caused milder disease in humans. Folklore maintained that human infection with cowpox conferred lifelong immunity to smallpox. In 1796 Jenner scientifically demonstrated that inoculation with material obtained from a milkmaid's cowpox lesions would result in immunity and protection from infection with smallpox when introduced by inoculation.[83] Jenner published his findings in 1798, and in 1801 he reported that 100,000 persons had been vaccinated in England. By the 1820s vaccination had become widespread throughout Britain and much of Europe. Although derivation of current vaccinia strains is uncertain, it is not a form of cowpox, and because Jenner lost his original material used for vaccination, the specific source of current vaccinia strains remains unknown.[83] The United States began regulating production of the vaccine in 1925. Since then, the New York City Board of Health strain of vaccinia has been used as the primary US vaccine strain. The WHO global vaccination program eventually led to smallpox eradication, with the last serially transmitted smallpox case reported in 1977. Routine vaccination of children in the United States ceased in 1971, and vaccination of hospital workers ceased in 1976. Vaccination of military personnel was continued because of Cold War concerns about its intentional use but eventually halted in 1989. The risk of bioterrorism prompted smallpox vaccination in at-risk military personnel and civilian healthcare workers to be resumed in 2003.[132,133]

During the WHO global eradication program, most of the human population received vaccinia virus by scarification. Although there were multiple manufacturers worldwide, and vaccine lots varied with respect to potency and purity, almost all vaccinia administered was derived from one of two lineages, the New York Board of Health and Lister strains.[2] Live vaccinia virus suspension was placed as a drop on the skin or drawn up by capillary action between the tines of a bifurcated needle; the nominal dose of live vaccinia was roughly 10^5 virions. Usually, primary vaccination is uneventful; following introduction into the skin, the virus replicates in basal layer keratinocytes, spreads cell-to-cell, and leads to discrete vesicle formation. Within a week, the vesicle evolves into a pustule surrounded by inflammatory tissue. This lesion scabs over within 10 to 14 days; eventually, the scab is shed. Vaccinees in the global campaign often experienced tender axillary lymph nodes, fever, and malaise for brief periods. Occasionally, however, complications arose with varying degrees of severity. Accidental transfer of vaccinia from the inoculation site was common, but of little consequence unless transferred to the eye. Generalized vaccinia, which involved systemic spread of the virus and eruption of multiple pocks at distant sites, was more serious. In individuals with eczema or atopic dermatitis, however, it sometimes led to extensive inflammation and secondary bacterial infection. More serious, life-threatening complications arose in vaccinees with defects in cell-mediated immunity; the vaccination site frequently enlarged to form an ulcer, secondary ulcers appeared, and the infection cleared slowly or not at all. The most serious event was postvaccinial encephalitis. Although rare, this condition was frequently fatal. Death occurred in approximately one in one million primary vaccinations.[134,135] Adverse events may be more frequent and severe if mass immunization were to be resumed in an unscreened general population that now includes transplant recipients on immunosuppressive drugs, HIV-infected individuals, and geriatric patients.

Recent Vaccination Campaigns. The requirement that any alternative vaccine must not be inferior to live vaccinia sets a high standard. The successful immunization or "take rate" has been greater than 95%, both historically and in a more recent series of more than 450,000 military vaccinees.[132] In this series, one case of encephalitis and 37 cases of myopericarditis were documented in a prescreened, healthy, young adult population. Although the incidence of myopericarditis was below the historical average and the cases were mild, this adverse event contributed to the general reluctance of the civilian healthcare population to accept vaccination.[133] Live (replicating) vaccinia immunization has also been used as postexposure prophylaxis and is believed effective if administered within 4 days of exposure. As a potential replacement for vaccine strategies used during the eradication campaign, a new vaccine was prepared in massive quantities (>300 million doses) by selection of plaque-purified progeny virus from the New York Board of Health strain (Dryvax, Wyeth Laboratories, Marietta, PA), which was amplified in VERO cell cultures. This vaccine is of greater purity and free of adventitious agents in comparison with its predecessor, which was prepared on calf skin. Phase I safety and immunogenicity trials for ACAM2000 indicate greater than 95% take rates and adverse events comparable to those of the predecessor vaccine.[136] ACAM2000 was approved for use in 2007 by the Food and Drug Administration (FDA), effectively replacing Dryvax as the vaccine of choice for licensed use. Additionally, this vaccine is currently being maintained as part of the Strategic National Stockpile.

ACAM2000 is no less reactogenic than Dryvax; therefore, vaccination remains limited to groups at the greatest risk of contracting orthopoxviruses.

Vaccination is performed with a bifurcated needle onto which the reconstituted vaccinia preparation has been drawn, using 15 jabs with enough strength to produce a visible trace of bleeding. The resulting vaccination lesion is then kept covered with a nonadherent and nonimpervious dressing. Care must be taken to prevent inadvertent inoculation of the vaccinee or others. In primary vaccinees, a papule forms within 5 days, developing into a vesicle on the 5th or 6th day postvaccination, which signifies a major reaction, or take. The vesicle subsequently becomes pustular, swelling subsides, and a crust forms, which comes off in 14 to 21 days. At the height of the primary reaction, known as the Jennerian response, regional lymphadenopathy usually occurs, which may be accompanied by systemic manifestations of fever and malaise. Primary vaccination with vaccine at a potency of 100 million pock-forming units per milliliter elicits a 97% response rate both by major reaction and neutralizing antibody response. Allergic sensitization to viral proteins can persist so that the appearance of a papule and redness may occur within 24 hours of revaccination, with vesicles occasionally developing within 24 to 48 hours. This allergic response peaks within 3 days and does not constitute a "major reaction or take." The immunological response occurring after 3 days is an accelerated but otherwise similar appearance of papule, vesicle, and/or pustule to that seen in the primary vaccination response. Revaccination is considered successful if a vesicular or pustular lesion or an area of definite palpable induration or congestion surrounding a central lesion (scar or ulcer) is present on examination at 6 to 8 days after revaccination.

The immunization of military and civilian individuals has provided an opportunity to study the nature of adverse events using modern tools of immunology. A strong association was established between adverse events and increased systemic cytokines, in particular, IFN-γ, tumor necrosis factor-α, interleukin-5, and interleukin-10.[137] Some researchers have speculated that cardiac events, although rare, may be related to dramatic alterations in cytokine profiles.

Protective immunity elicited by live vaccinia is thought to depend on a combination of humoral and cellular immune responses. Using a monkey model in which animals were immunized with vaccinia and challenged with monkeypox virus, Edghill-Smith et al showed that vaccinia specific B-cells are critical for protection.[138] Antibody depletion of B-cells (but not CD4+ or CD8+ T-cells) abrogated vaccinia-induced protection. Edghill-Smith et al have also shown that simian immunodeficiency virus compromised monkeys could withstand monkeypox infection if it was preceded by a dose of nonreplicating Modified Vaccinia Ankara (MVA) strain vaccinia, but they were not protected against monkeypox challenge when their CD4+ T-cell counts were < 300 mm.[138]

MVA is an alternative vaccine that has promise as a nonreplicating immunogen. MVA, which was used in Germany in the later stages of global eradication, was shown to be safe and immunogenic, but its protective efficacy has not been established in humans. MVA was generated by more than 500 serial passages in chick embryo fibroblasts, which resulted in multiple deletions and mutations and an inability to replicate efficiently in human and most other mammalian cells.[139] Ultrastructural examination of purified MVA reveals that most of the particles are enveloped; the host restriction occurs at a late stage of maturation. The presence of enveloped particles is believed to be important to the elicitation of protective immunity. Experimentally, MVA was demonstrated to protect monkeys against a monkeypox virus challenge after one or two doses of MVA or MVA followed by Dryvax.[140] Surprisingly, a single dose of MVA also protected when a challenge followed immunization by as little as 10 days, although protection was not absolute; a modest number of pocks and a low-level viremia occurred in the MVA recipients following challenge.

Aside from live and attenuated virus vaccines, a number of other approaches are being investigated. Most of these vaccine strategies involve the use of viral DNA or viral protein(s). Prime-boost strategies (ie, an initial vaccination with a DNA-based vaccine followed by a protein-based vaccine) have also been used. All of these strategies have shown some or significant promise in animal models; however, none of these have been approved by the FDA and are not currently being used as investigational new drugs.[141]

Outcome. Successful smallpox vaccination provides high-level immunity for the majority of recipients for 3 to 5 years followed by decreasing immunity. In Mack's review of importation cases in Europe from 1950 through 1972, he provided epidemiological evidence of some relative protection from death, if not from disease severity, in individuals who had been immunized more than 20 years before exposure. However, for the older population in particular, vaccination within 10 years of exposure did not prevent all cases but did prevent some smallpox deaths.[142] Multiple vaccinations are thought to produce more long-lasting immunity. Vaccination has been effective in preventing disease in 95% of vaccinees.[143] Vaccination was also shown to prevent or substantially reduce the severity of infection when given as a secondary prophylaxis within a few days of exposure.[2]

Contraindications. Smallpox vaccination is contraindicated in the preoutbreak setting for individuals with the following conditions or those having close contact with individuals with the following conditions:

- a history of atopic dermatitis (eczema);
- acute, chronic, or exfoliative skin conditions that disrupt the epidermis;
- pregnancy or the possibility of becoming pregnant; or
- a compromised immune system as a consequence of HIV infection, acquired immunodeficiency syndrome, autoimmune disorders, cancer, radiation treatment, immunosuppressive therapy, or other immunodeficiencies.

Additional relative contraindications for potential vaccinees, but not close contacts, are smallpox vaccine-component allergies, moderate or severe acute intercurrent infections, topical ophthalmologic steroid medications, age younger than 18, and maternal breastfeeding. A history of Darier's disease and household contact with active disease also are contraindications for vaccination.[6]

Adverse Events. Vaccinia can be transmitted from a vaccinee's unhealed vaccination site to other persons by close contact and the same adverse events as with intentional vaccination can result. To avoid inadvertent transmission, vaccinees should wash their hands with soap and water or use antiseptic hand rubs immediately after touching the vaccination site and after dressing changes. Vaccinia contaminated dressings should be placed in sealed plastic bags and disposed in household trash.[144]

Adverse reactions to smallpox vaccination are diagnosed by a clinical examination. Most reactions can be managed with observation and supportive measures. Self-limited reactions include fever, headache, fatigue, myalgia, chills, local skin reactions, nonspecific rashes, erythema multiforme, lymphadenopathy, and pain at the vaccination site. Adverse reactions that require further evaluation and possible therapeutic intervention include inadvertent inoculation involving the eye, generalized vaccinia, eczema vaccinatum, progressive vaccinia, postvaccinial central nervous system disease, and fetal vaccinia.[6,145]

Inadvertent inoculation generally results in a condition that is self-limited unless it involves the eye or eyelid, which requires an ophthalmologist's evaluation. Topical treatment with trifluridine (Viroptic, Glaxo/Smith/Kline, Brentford, Middlesex, United Kingdom) or vidarabine (ViraA, King Pharmaceuticals, Bristol, TN) is often recommended, although the FDA does not specifically approve the treatment of ocular vaccinia for either of these drugs. Most published experience is with use of vidarabine, but this drug is no longer manufactured.[146]

Generalized vaccinia is characterized by a disseminated maculopapular or vesicular rash, frequently on an erythematous base, and typically occurring 6 to 9 days after primary vaccination. Treatment with vaccinia immune globulin (VIG) is restricted to those who are systemically ill or have an immunocompromising condition or recurrent disease that can last up to a year. Contact precautions should be used to prevent further transmission and nosocomial infection.[6]

Eczema vaccinatum occurs in individuals with a history of atopic dermatitis, regardless of current disease activity, and can be a papular, vesicular, or pustular rash. This rash may be generalized, or localized with involvement anywhere on the body, with a predilection for areas of previous atopic dermatitis lesions. Mortality ranges from 17% to 30% and is reduced by use of VIG. Contact precautions should be used to prevent further transmission and nosocomial infection.[6]

Progressive vaccinia is a rare, severe, and often fatal complication of vaccination that occurs in individuals with immunodeficiency conditions and is characterized by painless progressive necrosis at the vaccination site with or without metastases to distant sites.

This condition carries a high mortality rate; therefore, progressive vaccinia should be aggressively treated with VIG, intensive monitoring, and tertiary medical center level support. Persons with the following conditions are at the highest risk:

- congenital or acquired immunodeficiencies;
- HIV infection/acquired immunodeficiency syndrome;
- cancer;
- autoimmune disease;
- immunosuppressive therapy; or
- organ transplant.

Anecdotal experience has shown that despite treatment with VIG, individuals with cell-mediated immunity defects have a poorer prognosis than those with humoral defects. Infection control measures should include contact and respiratory precautions to prevent transmission and nosocomial infection.[6]

Central nervous system disease, which includes postvaccinial encephalopathy and postvaccinial encephalomyelitis, occurs rarely after smallpox vaccination. Postvaccinial encephalopathy occurs more frequently, typically affects infants and children younger than age 2, and reflects vascular damage to the central nervous system. Symptoms that typically occur 6 to 10 days postvaccination include

seizures, hemiplegia, aphasia, and transient amnesia. Histopathologic findings include cerebral edema, lymphocytic meningeal inflammation, ganglion degeneration, and perivascular hemorrhage. Patients with postvaccinial encephalopathy who survive can be left with cerebral impairment and hemiplegia. Postvaccinial encephalomyelitis, which affects individuals who are age 2 or older, is characterized by abrupt onset of fever, vomiting, malaise, and anorexia occurring approximately 11 to 15 days postvaccination. Symptoms progress to amnesia, confusion, disorientation, restlessness, delirium, drowsiness, and seizures. The cerebral spinal fluid has normal chemistries and cell count. Histopathology findings include demyelization and microglial proliferation in demyelinated areas, with lymphocytic infiltration but without significant edema. The cause for central nervous system disease is unknown, and no specific therapy exists. Therefore, intervention is limited to anticonvulsant therapy and intensive supportive care. Fetal vaccinia, which results from vaccinial transmission from mother to fetus, is a rare but serious complication of smallpox vaccination during or immediately before pregnancy.[6]

In the Department of Defense 2002–2003 vaccination program involving 540,824 vaccinees,[66] symptomatic cases of myopericarditis were reported, for a rate of 1.2 per 10,000. Mean time from vaccination to evaluation for myopericarditis was 10.4 days, with a range of 3 to 25 days. Reports of myocarditis in vaccinees in 2003 raised concerns of carditis and cardiac deaths in individuals undergoing smallpox vaccination. That year, 21 cases of myo/pericarditis of 36,217 vaccinees were reported, with 19 (90%) occurring in revaccinees. The median age of those affected was 48, and they were predominantly women. Eleven of the individuals were hospitalized, but there were no fatalities. Of the 540,824 total vaccinees over the 2 years, 449,198 were military personnel (the rest were civilians), and of these there were 37 cases, for an occurrence rate of 1 per 12,000 vaccinees.[131] Ischemic cardiac events including fatalities have also been reported as a consequence of the use of vaccinia vaccine (Dryvax) during the campaign. Although no clear association has been found, history of ischemic heart disease and significant cardiac risk pose relative contraindications for smallpox vaccination. Consequently, individuals with a history of myocarditis, pericarditis, or ischemic heart disease should refrain from vaccination.[147,148]

Smallpox Biothreat Policy. In a smallpox release from a bioterrorist event, individuals would be vaccinated according to the current national policy, which recommends initial vaccination of higher risk groups (individuals directly exposed to the release and those with close contact to smallpox patients) and medical and emergency transport personnel. Vaccination of the general population would then be extended in concentric rings around the initial cases to impede the spread. There are no absolute contraindications to vaccination for individuals with high-risk exposure to smallpox. Persons at the greatest risk of complications of vaccination are those for whom smallpox infection poses the greatest risk. If relative contraindications exist for an individual, the risks must be weighed against the risk of a potentially fatal smallpox infection.

Postexposure prophylaxis with vaccine offers protection against smallpox but is untried in human infections with other orthopoxviruses.[2] Despite a lack of hard evidence in humans, postexposure vaccination is likely efficacious against other orthopoxviruses, and during the 2003 US monkeypox outbreak the CDC recommended vaccination of potentially exposed persons.[93]

Treatment

Passive Immunization

VIG is available from the CDC as an investigational new drug in two formulations: intramuscular and intravenous. VIG may be beneficial in treating some of the adverse effects associated with vaccination. VIG has no proven benefit in smallpox treatment, and its efficacy in treatment of monkeypox infections is unknown. Monoclonal antibodies have been shown to be beneficial in animal models under certain conditions, but this concept has not yet been sufficiently developed for efficacy testing in humans.

Antiviral Drugs

The introduction of monkeypox virus to the United States, the endemic nature of monkeypox virus in certain regions of Africa, and the continued threat of an act of bioterrorism with monkeypox or variola indicate the need for anti-*Orthopoxvirus* therapeutic drugs. In addition, a therapeutic would be useful for the treatment of adverse events associated with vaccination. The only FDA approved antiviral drug available for treating orthopoxviruses is cidofovir. However, it is approved for treatment of cytomegalovirus in HIV patients so it can only be offered for treatment of orthopoxvirus infections under emergency use protocols maintained by both the Department of Health and Human Services and the Department of Defense.[149]

The elaborate replication strategy of poxviruses offers a number of potential targets for therapeutic intervention.[150] Initial studies to identify effective antiviral agents for orthopoxviruses tested drugs developed for other viruses that share similar molecular targets.[149] The effort to discover effective drugs against DNA

viruses initially focused on treatment of herpesvirus infections. The discovery of acyclovir led to practical therapy and a better understanding of the importance of viral and cellular enzymes involved in phosphorylation of acyclovir to acyclovir triphosphate, the active chemical entity. Acyclovir failed to inhibit cytomegalovirus because unlike the thymidine kinase of herpes simplex, cytomegalovirus thymidine kinase lacked the appropriate specificity, which was overcome by synthesis of a series of phosphorylated analogues using a stable phosphonate bond. The most promising candidate using this approach was cidofovir, which is a dCMP analog.[151] Cidofovir is licensed for treatment of cytomegalovirus-associated retinitis under the trade name Vistide (Gilead Sciences Inc, Foster City, CA), and inhibits the cytomegalovirus DNA polymerase, a target shared with the poxviruses. Cidofovir has been demonstrated to protect nonhuman primates against severe disease in both the monkeypox and variola nonhuman primate models, when administered within 48 hours of intravenous or respiratory exposure to the virus.[152–157] Although the drug formulation used in these studies has been criticized for requiring intravenous administration, patients with advanced disease would already be receiving intravenous fluids as part of their supportive care, and once weekly or every other day cidofovir administration would not significantly increase the healthcare burden. Cidofovir has been associated with nephrotoxicity; therefore, careful attention to fluid management is important and patient hydration and coadministration of probenecid is required.

Cidofovir requires bolus dosing to allow drug entry into cells by pinocytosis; however, bolus dosing results in transiently high concentrations in the kidney. For this reason, an oral formulation with lower toxicity is more desirable. CMX001 is a lipid conjugate of cidofovir with a 1-0-hexadecyl-oxypro-pyl (HDP) covalently linked to the nucleotide analogue. Attachment of the lipid moiety allows CMX001 to be taken up into cells through lysophosphatidylcholine (LPC) uptake pathways,[158] which results in lower toxicity and increased bioavailability. This formulation dramatically reduced transient drug levels in the kidney and eliminated nephrotoxicity in toxicology studies using mice.[159] CMX001 has also demonstrated protection in mouse and rabbit models of orthopoxvirus infection. Demonstrating efficacy of CMX001 in nonhuman primates is not possible because of the higher oxidative metabolism in monkeys. However, both cidofovir and CMX001 produce the same antiviral product in vivo (cidofovir diphosphate), allowing cidofovir to be used as a surrogate. Increased oxidative metabolism is not observed in humans. Although an oral formulation of cidofovir is not yet available for human use, it is in phase I/II clinical trials and is used under emergency authorization for the treatment of systemic adenovirus infections of pediatric hematopoietic stem cell transplant recipients.[160]

An alternative approach to identifying compounds with anti-*Orthopoxvirus* activity was the use of a high-throughput screen using vaccinia and cowpox virus. More than 300,000 compounds were evaluated and several potent lead structures were identified for optimization and evaluation against vaccinia, monkeypox, and variola viruses.[161] From this effort ST-246 [4-trifluoromethyl-*N*-(3,3a,4,4a,5,5a,6,6a-octahydro-1,3-dioxo-4,6-ethenocycloprop[*f*]isoindol-2(1*H*)-yl)-benzamide] was identified and is under advanced development by the Biomedical Advanced Research and Development Authority. ST-246 is both potent (EC_{50} < 0.010 μM), selective (CC_{50} > 40 mM), and active against multiple orthopoxviruses, including monkeypox, camelpox, cowpox, ectromelia (mousepox), vaccinia, and variola viruses in vitro; and against monkeypox, variola, cowpox, vaccinia, and ectromelia in vivo.[161] The viral target of ST-246 is the F13L (homologue) gene product p37. This viral phospholipase plays a critical role in egress of viral particles from the host cell and the inhibition of this process with ST-246 reduced extracellular virus by 10-fold.[161] ST-246 has demonstrated efficacy in multiple animal models of orthopoxvirus infection both prophylactically and therapeutically, and more importantly, it has prevented morbidity and mortality against monkeypox virus and prevented mortality from variola virus in nonhuman primates.[162] In addition, the compound was well tolerated by human subjects after daily oral administration for 21 consecutive days.[163] ST-246 has been placed in the Strategic National Stockpile, but FDA has not approved it.

Cidofovir, ST-246, CMX001, and VIG have been used successfully in combination to treat the adverse effects of vaccination. In a recent case, vaccinia virus mutants resistant to ST-246 were isolated from a marine who developed progressive vaccinia following vaccination and was subsequently treated with ST-246, CMX001, and VIG.[164] The patient recovered, but it highlighted the importance of combination therapy; and as stated previously, VIG is not in development as an antiviral against monkeypox or variola virus.

Myriad alternative approaches to orthopoxvirus treatment, such as interferon mimetics, interferon beta, RNAi, mixantrone, and terameprocol (to name a recent few), are under investigation. These compounds have shown varying degrees of success in a wide range of test systems; however, none of them has reached the stage of clinical development for use as an anti-*Orthopoxvirus* treatment.[157,165–170]

SUMMARY

Smallpox no longer causes human disease thanks to the dedicated efforts of public health officials who participated in the WHO smallpox eradication program. Although the former Soviet Union participated in the eradication program, it is believed that the Soviets continued developing smallpox for biowarfare into the 1980s. The Soviet Union is dissolved and its offensive program has been dismantled, but the institutions and technology that developed this and other offensive weapons systems remain. Because the submission and destruction of smallpox virus stores was a voluntary program, it cannot be ascertained with certainty that smallpox viruses do not exist outside US and Russian storage facilities. Since the sequence of several variola isolates is known to a high degree of certainty, it is technically possible to generate viable virus either by modification of a closely related virus such as camelpox, or chemical synthesis using increasingly powerful automated equipment.

The potential threat from smallpox specifically and orthopoxvirus infections in general will expand as the technology to create these viruses becomes increasingly available in laboratories around the world. Furthermore, scientists have been successful in making orthopoxviruses more virulent through genetic manipulation. The biodefense community has made considerable progress in developing new drugs such as ST-246 and CMX001 for treatment of orthopoxvirus infections and safer vaccines. There is still no approved treatment for smallpox; however, FDA approved ACAM2000 as a smallpox vaccine in 2007. MVA, although not FDA approved, is placed in the Strategic National Stockpile.

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Chapter 25

EMERGING INFECTIOUS DISEASES AND FUTURE THREATS

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SUMMARY

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INTRODUCTION

What Are Emerging Infectious Diseases?

Infectious diseases have caused the deadliest pandemics in recorded human history. Some of these have included the Black Death (bubonic plague resulting in 25–40 million deaths), the 1918–1919 influenza pandemic ("Spanish Flu" resulting in an estimated 50 million deaths), and the ongoing human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) pandemic (resulting in 35 million deaths to date).[1] Emerging infectious diseases, as defined in the landmark report by the Institute of Medicine in 1992, are those diseases whose incidence has increased within the past 20 years or whose incidence threatens to increase in the near future.[2] Although some "emerging" diseases have been now recognized for more than 20 years (eg, HIV/AIDS, Lyme disease or Legionnaires' disease), their importance has not diminished, and the factors associated with their emergence are still relevant. Emerging infections include those that are recognized in their host (humans, animals, or plants) for the first time or caused by new or newly described agents. Reemerging diseases are diseases that historically have infected humans, but appear in new locations, or whose incidence had previously declined, but now are increasing. In addition, this definition includes organisms that are developing antimicrobial resistance and established chronic diseases with a recently discovered infectious origin.

Factors That Contribute to Emerging Infectious Diseases

Many factors contribute to the emergence of new diseases. In the United States, in particular, these factors include increasing population density and urbanization; immunosuppression (resulting from aging, malnutrition, cancer, or infection with HIV); changes in land use (eg, deforestation, reforestation, and fragmentation), climate, and weather; international travel and commerce; microbial or vector adaptation and change (mutations resulting in drug/pesticide resistance or increased virulence).[2] Internationally, many of these factors also hold true; however, many developing countries also have to deal with war, political instability, inadequate healthcare, and basic sanitation needs.

The numerous examples of novel infections originating from animal species (ie, zoonoses) suggest that animals are an important source of emerging diseases.[3] Although it is controversial whether HIV/AIDS should be considered a zoonotic disease,[4] it is now clear that both HIV-1 and HIV-2 had zoonotic origins.[5–7] In addition, as was observed with the 2003 outbreak of monkeypox in the United States, increasing trade in exotic animals for pets has led to increased opportunities for pathogens to "jump" from animal reservoirs to humans. The use of exotic animals (eg, Himalayan palm civets) for food in China and the close aggregation of numerous animal species in public markets may have led to the emergence of the 2002–2003 outbreak of severe acute respiratory syndrome (SARS).[8]

Many of the viruses or bacteria that cause concern as potential bioweapons are considered emerging pathogens, and most are also of zoonotic origin. In particular, some of these agents have appeared in new geographical locations where they were not previously seen (eg, the sudden occurrence of monkeypox in the midwest of the United States in 2003, and the largest recorded outbreak of Marburg hemorrhagic fever in Angola in 2005). In some cases, the specific use of a pathogen in an act of bioterrorism could classify that pathogen as an emerging or reemerging disease agent, as was the case for *Bacillus anthracis* during the 2001 anthrax attacks in the United States. Through increasingly accessible molecular biology techniques, completely new organisms—or significant modification of existing ones—can now be made in the laboratory (ie, synthetic biology). The use of these methods is beneficial and necessary for modern biomedical research to proceed. However, the same methods and techniques can be used for nefarious purposes and, along with naturally occurring emerging infections, represent significant future threats to both military and civilian populations.

More than 20 years after the Institute of Medicine Report, much progress on emerging infectious diseases has been made, including a greater awareness; use of next-generation sequencing for the characterization of pathogens, vectors, and their hosts and for enhanced diagnostics; and increased laboratory infrastructure including additional biocontainment laboratories (ie, biosafety level 3 laboratories and biosafety level 4 laboratories) to safely work with these pathogens.[9] Despite this progress, new diseases continue to emerge. This continual emergence of new infectious diseases poses a continuing challenge, requiring constant surveillance, the ability to promptly respond with new diagnostics and new vaccines and drugs, and ongoing research into the basic biology of novel pathogens.

EMERGING BACTERIAL DISEASES

Waterborne Diseases

Emerging waterborne diseases constitute a major health hazard in both developing and developed countries. From 2007 to 2008, 48 disease outbreaks associated with contaminated drinking water were reported in the United States, resulting in 4,128 ill people and 3 deaths.[10] During this same time, more than 13,966 cases of illness were associated with 134 recreational water-associated outbreaks of disease.[11] Although these numbers represent disease caused by a range of pathogenic organisms (ie, viruses, bacteria, parasites), the majority (58%) of drinking water-associated outbreaks were caused by bacterial pathogens. Bacterial pathogens associated with drinking water disease outbreaks included *Legionella* (12 outbreaks), *Campylobacter* (4 outbreaks), *Salmonella* (3 outbreaks), *Escherichia coli* O157:H7 and *Providencia* (1 outbreak each).[10] Bacterial pathogens were responsible for 21% of the outbreaks of disease associated with recreational water exposure. Diseases associated with exposure to recreational water are more diverse than those associated with drinking water and include acute gastrointestinal illness, acute respiratory illness, and dermatologic illness. Accordingly, the list of bacterial pathogens responsible is more diverse and includes *E coli* O157:H7, *Shigella sonnei*, *Legionella*, *Plesiomonas shigelloides*, and *Vibrio vulnificus*. Dermatitis outbreaks were most often attributed to *Pseudomonas*, primarily *P aeruginosa*.[12] Internationally, cholera (caused by *Vibrio cholerae*) is still a major killer, as demonstrated by recent large outbreaks in Sierra Leone, Zimbabwe, and Democratic Republic of Congo in Africa resulting in more than 120,000 cases combined and more than double that number of cases in Haiti following a massive earthquake in 2010 (see Cholera and *Vibrio cholerae*).

Cholera and Vibrio cholerae

Cholera is one of the most rapidly fatal diseases known, capable of killing within 12 to 24 hours after onset of diarrhea. The World Health Organization (WHO) estimates 3 to 5 million cholera illnesses and up to 130,000 deaths occur globally each year.[13] Cholera accounts date back to Hippocrates.[14] Seven worldwide cholera pandemics have occurred. An 1892 cholera outbreak in Hamburg, Germany, affecting 17,000 people and causing 8,605 deaths, was attributed to the inadvertent contamination of the city's water supply by bacteriologists studying the pathogen.[15] This event underscores the potential for cholera to cause widespread illness where water is not disinfected with a modern bacteriocide such as chlorine.[15]

In 1991, after almost a century without cholera, outbreaks in Latin America resulted in about 400,000 cases of cholera and more than 4,000 deaths.[16] Off the Peruvian coast, a significant correlation existed between cholera incidence and elevated sea surface temperature from 1997 to 2000, which included the 1997 to 1998 El Niño event.[17] Some people believe that the eighth worldwide pandemic began in 1992.[18] During 2011, 58 countries reported a total of 589,854 cases including 7,816 deaths from cholera.[13] Cholera cases in the United States have decreased to about 10 cases per year from 1995 through 2009; however, 42 cases were reported in 2011.[13] Most of these cases were either travel-associated or associated with consumption of undercooked seafood harvested along the Gulf Coast.

Cholera occurs through fecal-oral transmission brought about by deterioration of sanitary conditions. Epidemics are strongly linked to the consumption of unsafe water, poor hygiene, poor sanitation, and crowded living conditions (Figure 25-1). Water or food contaminated by human waste is the major vehicle for disease transmission. Cholera transmission is thought to require 10^3 organisms to exert an effect in the gut, with 10^{11} organisms as a minimum infective dose needed to survive stomach acid.[19]

Before 1992, all previous cholera pandemics were caused by the *V cholerae* serogroup O1 (classical) or El Tor biotypes. Large outbreaks in 1992 resulted from transmission of a previously unknown serogroup, *V cholerae* O139, which has since spread from India and Bangladesh to countries throughout Asia, including Pakistan, Nepal, China, Thailand, Kazakhstan, Afghanistan, and Malaysia.[20]

Enterotoxin produced by *V cholerae* O1 and O139 can cause severe fluid loss from the gut. In severe cases, profuse watery diarrhea, nausea, and vomiting can lead to rapid dehydration, acidosis, circulatory collapse, and renal failure. Successful treatment of cholera patients depends on rapid fluid and electrolyte replacement. Antimicrobial therapy can also be useful.

Mixed success has been obtained with cholera vaccines. Historically, live attenuated vaccines have been more effective than killed whole-cell vaccines.[21] No licensed cholera vaccines are available in the United States.



Figure 25-1. Typical conditions that can lead to a cholera epidemic. This photograph was taken in 1974 during a cholera research and nutrition survey amidst floodwaters in Bangladesh.
Photograph: Courtesy of Dr Jack Weissman, Centers for Disease Control and Prevention Public Health Image Library.

On January 12, 2010, a magnitude 7.0 earthquake decimated the island nation of Haiti, leaving a quarter of a million people dead, 300,000 injured, and 2 million homeless. Nine months later, in October, a cholera outbreak was confirmed in Haiti,[22] indicating the first occurrence of cholera in Haiti in at least 100 years. Some have suggested that cholera may never have been in Haiti before 2010.[23] Based on epidemiological data, the cholera outbreak began in the upstream region of the Artibonite River (Figure 25-2).[24] The presumed first case was a 28-year-old man with a history of severe untreated psychiatric disease.[25] The patient had a history of wandering nude through town throughout the day and both bathing in and drinking the water from the Latem River, one of the tributaries of the Artibonite River. On October 12, 2010, he developed acute onset of profuse watery diarrhea. In less than 24 hours after the onset of symptoms, he died at home without seeking medical attention. The first hospitalized case of cholera in Haiti occurred at the Mirebalais Government Hospital on October 17, 2010.[24]

By mid-November, cholera had spread to every part of the country and to neighboring Dominican Republic, and by mid-December a total of 121,518 cases of cholera, resulting in 63,711 hospitalizations and 2,591 deaths, had been reported from Haiti.[22] The cholera outbreak in Haiti has continued since 2010 with more than 734,983 cases and 8,761 deaths as of April 3, 2015, according to the Pan American Health Organization.[26]

The outbreak strain was identified as *V cholerae* O1, serotype Ogawa, biotype El Tor.[27] Whole genome DNA sequencing and epidemiological analysis confirmed that the outbreak strain was inadvertently introduced into Haiti by United Nations security forces from Nepal.[28–30]

A cholera outbreak was reported in Kathmandu (Nepal's capital city) on September 23, 2010, shortly before troops left for Haiti.[31] The first cholera cases in Haiti came from a village named Meye, located 150 meters downstream from the Nepalese military camp.[24,30] Taken together, evidence strongly supports the conclusion that the United Nations military camp, housing the Nepalese peacekeeping troops in Meye, was the source of the Haitian cholera epidemic. These findings led to considerable political unrest and have forever changed the global response to natural disasters. In late 2013, survivors and family members of the nearly 700,000 Haitians who contracted cholera sued the United Nations, accusing them of covering up its role in starting the cholera outbreak in Haiti. In early 2015, a US federal judge ruled that the Haitians could not sue the United Nations because the organization has legal



Figure 25-2. The Artibonite River is the longest and most important river in Haiti. It forms part of the international border between Haiti and the Dominican Republic and empties into the Gulf of Gonâve. It is believed that the 2010 cholera outbreak began in the upstream region of this river.
Photograph: Courtesy of Kendra Helmer, US Agency for International Development.

immunity against lawsuits. In August 2016, the Court upheld the United Nations' immunity from claims (http://www.ijdh.org/cholera/cholera-litigation/).

Other Vibrioses

In recent years, some noncholera vibrios have acquired increasing importance because of their association with human disease. More than 70 members are in the family *Vibrionaceae*, 12 of which have been isolated from human clinical specimens and apparently are pathogenic for humans.[32] Vibrio species are primarily aquatic and common in marine and estuarine environments and on the surface and in the intestinal tracts of marine animals. *V parahemolyticus* and *V vulnificus* are halophilic vibrios commonly associated with consumption of undercooked seafood. Diarrhea, cramping, nausea, vomiting, fever, and headache are commonly associated with *V parahemolyticus* infections.

Cases of diarrhea related to seafood consumption increased worldwide with the emergence of pandemic strain O3:K6, which was originally observed in Southeast Asia.[33] *V vulnificus* is the most common source of vibrio infections in the United States resulting in gastrointestinal symptoms similar to *V parahemolyticus*, but may also lead to ulcerative skin infections if open wounds are exposed to contaminated water. Septicemia can occur in those infected with *V vulnificus* who are immunosuppressed or have liver disease or chronic alcoholism, and septicemic patients can have a mortality rate of up to 50%. In most cases the disease begins several days after the patient has eaten raw oysters. Other human pathogenic species include *V mimicus*, *V metschnikovii*, *V cincinnatiensis*, *V hollisae*, *V damsela*, *V fluvialis*, *V furnissii*, *V alginolyticus*, and *V harveyi*; most of these have been associated with sporadic diarrhea, septicemia, and wound infections.[32]

Legionellosis

Legionnaires' disease was first recognized in 1976 after a large outbreak of severe pneumonia occurred among attendees at a convention of war veterans in Philadelphia. A total of 182 people, all members of the Pennsylvania American Legion, developed an acute respiratory illness, and 29 individuals died from the disease.[34] The cause of the outbreak remained a mystery for 6 months until the discovery by Joseph McDade, a Centers for Disease Control and Prevention (CDC) microbiologist, of a few gram-negative bacilli, subsequently named *Legionella pneumophila*,[35] in a gram stain of tissue from a guinea pig inoculated with lung tissue from a patient who died from the disease.[36] Using the indirect immunofluorescence assay, McDade showed that the sera of patients from the convention mounted an antibody response against the newly isolated bacterium,[36] marking the discovery of a whole new family of pathogenic bacteria. Retrospective analysis, however, showed that outbreaks of acute respiratory disease from as far back as 1957 have now been attributed to *L pneumophia*.[37,38] The earliest recorded isolate of a *Legionella* species was recovered by Hugh Tatlock in 1943 during an outbreak of Fort Bragg fever.[39,40]

Legionnaires' disease is normally acquired by inhalation or aspiration of *L pneumophila* or other closely related *Legionella* species. Water is the major reservoir for legionellae, and the bacteria are found in freshwater environments worldwide. Legionnaires' disease has been associated with various water sources where bacterial growth is permitted, including cooling towers,[41] whirlpool spas,[42] and grocery store mist machines.[42] The association between a portable shower and nosocomial legionellosis was demonstrated more than 30 years ago.[43] The most common source of legionellosis in hospitals is from the hot water system,[44] and sustained transmission of Legionnaires' disease in the hospital environment can be difficult to control.[42] Community-acquired legionellosis is thought to account for most infections.[45] An Italian survey of household hot water systems in 2000 found bacterial contamination, with *Legionella* species in 23% of the homes and *Pseudomonas* species in 38%. One *Legionella* species, *L longbeachae*, has been associated with disease transmission from potting soil.[16]

Legionnaires' disease is an acute bacterial illness that initially presents with anorexia, malaise, myalgia, and headache, with a rapidly rising fever and chills. Temperatures commonly reach 102°F to 105°F and are associated with nonproductive cough, abdominal pain, and diarrhea. The disease may eventually progress to respiratory failure and has a case-fatality rate as high as 39% in hospitalized cases. Nonpneumonic legionellosis, or Pontiac fever, occurs after exposure to aerosols of water colonized with *Legionella* species.[46–48] Attack rates after exposure to an aerosol-generating source, which often range from 50% to 80%, are exceptionally high. After a typical asymptomatic interval of 12 to 48 hours after exposure, patients note the abrupt onset of fever, chills, headache, malaise, and myalgias. Pneumonia is absent and those who are affected recover in 2 to 7 days without receiving specific treatment.[49]

Legionella is now recognized around the world as an important cause of community-acquired and hospital-acquired pneumonia, occurring both sporadically and in outbreaks. Although 90% of *Legionella* infections in humans are caused by *L pneumophila*, there are 50 named species of *Legionella*, with approximately 20

known to cause human infections.[50] Some unusual strains of bacteria, which infect amoebae and have been termed *Legionella*-like amoebal pathogens (LLAPs), appear to be closely related to *Legionella* species on the basis of 16S ribosomal RNA gene sequencing.[51,52] Three LLAP strains are now named *Legionella* species[53]; one of them, LLAP-3, which was first isolated from the sputum of a patient with pneumonia by coculture with amoebae, is considered a human pathogen.[54]

Foodborne Diseases

More than 200 diseases are transmitted through food, including illnesses resulting from viruses, bacteria, parasites, toxins, metals, and prions. In the United States, the burden of foodborne illness is estimated at approximately 76 million illnesses, 325,000 hospitalizations, and 5,000 deaths each year.[55] Among the bacterial pathogens estimated to cause the greatest number of US foodborne illnesses are *Campylobacter*, *Salmonella*, *Shigella*, *Clostridium*, and *Staphylococcus*.[55] Emerging bacterial illnesses include *E coli* O157:H7 and other enterhemorrhagic and enterotoxigenic *E coli*, as well as antibiotic resistant bacteria. Many of the pathogens of greatest concern today (eg, *C jejuni*, *E coli* O157:H7, *Listeria monocytogenes*, *Cyclospora cayetanensis*) were not recognized as causes of foodborne illness just 20 years ago. Other rare pathogens, such as *E coli* O104:H4, recently emerged as a cause of a foodborne outbreak of hemolytic uremic syndrome (HUS) in northern Germany resulting in more than 4,000 cases and 54 deaths (see section on Disease Caused by *Escherichia coli* O104:H4).

The majority of gastrointestinal illnesses are caused by foodborne agents not yet identified. It is estimated that 62 million foodborne-related illnesses and 3,200 deaths occur in the United States each year from unknown pathogens.[55] *Bacillus anthracis*, although rarely seen as a gastrointestinal illness in the United States, has become a concern since cases occurred in 2000 and 2009 (see next section). Even in areas of the world where gastrointestinal anthrax is more common, the oropharyngeal form is underreported because physicians are unfamiliar with it.[56] Unreported foodborne disease, deaths from unknown food agents,[57] and chronic sequelae[58] may be a huge unrecognized burden of illness.

Gastrointestinal Anthrax

Bacillus anthracis is the causative agent of anthrax, a naturally occurring zoonotic disease. The greatest bioweapons threat from anthrax is through aerosol dispersion and subsequent inhalation of concentrated spores (for more details, see Chapter 6, Anthrax). Gastrointestinal anthrax, however, is contracted through the ingestion of *B anthracis* spores in contaminated food or water. This form of the disease occurs more commonly than inhalational anthrax in the developing world, but it is rare in the United States and other developed nations.[56,59] In one large outbreak in Uganda, 155 villagers ate the meat of a zebu (bovine) that had died of an unknown disease. Within 15 to 72 hours, 143 (92%) persons developed presumed anthrax. Of these, 91% had gastrointestinal complaints and 9% oropharyngeal edema; nine of them—all children—died within 48 hours of illness onset.[59]

Although rare in the United States, gastrointestinal anthrax does occur naturally, and anthrax-contaminated meat was found to be associated with gastrointestinal illness in Minnesota in 2000.[16] Another case occurred in 2009 from exposure to animal-hide drums.[22] Purposeful contamination of food or water is possible, but it would require a high infective dose. Misdiagnosis of gastrointestinal anthrax may lead to a higher mortality than other forms of anthrax; thus awareness of this disease remains important in anthrax-endemic areas and in the setting of possible bioterrorism.

Disease Caused by **Campylobacter jejuni**

Campylobacter was first identified in 1909 (then called *Vibrio fetus*) from the placentas and aborted fetuses of cattle. The organism was not isolated from humans until nearly 40 years later when it was found in the blood of a pregnant woman who had an infectious abortion in 1947.[60] *Campylobacter jejuni* (Figure 25-3),

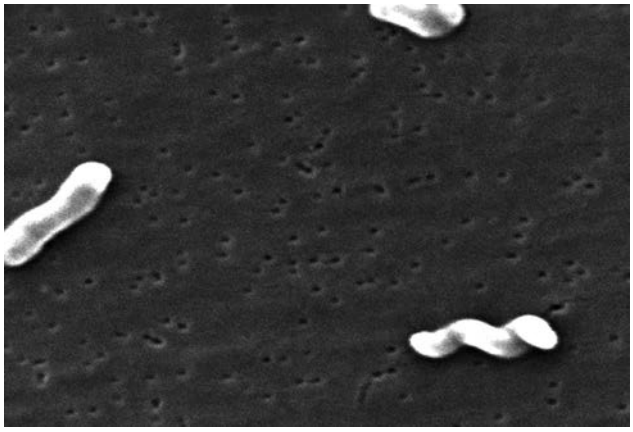


Figure 25-3. Scanning electron microscope image of *Campylobacter jejuni* illustrating its corkscrew appearance. Magnification ×11,734.
Photograph: Courtesy of Janice Carr, Centers for Disease Control and Prevention Public Health Image Library.

along with *C coli*, have been recognized as agents of gastrointestinal infection since the late 1970s. Today, *C jejuni* is considered the most commonly reported foodborne bacterial pathogen in the United States, affecting 2.4 million persons annually.[61] Campylobacteriosis is an enteric illness of variable severity including diarrhea (which may be bloody), abdominal pain, malaise, fever, nausea, and vomiting occurring 2 to 5 days after exposure. Many infections are asymptomatic; however, infection with this pathogen has also been associated with development of Guillain-Barré syndrome and arthritis.[62,63] Infants are more susceptible to *C jejuni* infections upon first exposure.[64] Persons who recover from *C jejuni* infection develop immunity. Poultry colonized with *Campylobacter* species is a major source of infections for humans.[65–67] The reported incidence of *Campylobacter* species on poultry carcasses has varied, but has been as high as 100%.[66]

Several virulence properties, including motility, adherence, invasion, and toxin production, have been recognized in *C jejuni*.[68] Along with several other enteric bacteria, *C jejuni* produces a toxin called cytolethal distending toxin that works by a completely novel mechanism; mammalian cells exposed to the toxin distend to almost 10 times their normal size from a molecular blockage in their cell cycle.[69] Although cytolethal distending toxin is the best characterized *Campylobacter* toxin, its role in the pathogenesis of human campylobacteriosis is still unclear.[70]

Because illness from *Campylobacter* infection is generally self-limited, no treatment other than rehydration and electrolyte replacement is generally recommended. However, in more severe cases (ie, high fever, bloody diarrhea, or septicemia), antibiotic therapy can be used to shorten the duration of symptoms if it is given early in the illness. Because infection with *C jejuni* in pregnant women may have deleterious effects on the fetus, infected pregnant women receive antimicrobial treatment. Erythromycin, the drug of choice for *C jejuni* infections, is safe, lacks serious toxicity, and is easy to administer. However, most clinical trials performed in adults or children have not found that erythromycin significantly alters the clinical course of *Campylobacter* infections.[71,72] Other antimicrobial agents, particularly the quinolones (eg, fluoroquinolones such as ciprofloxacin) and newer macrolides including azithromycin are also being used. Unfortunately, as the use of fluoroquinolones has expanded (especially in food animals), the rate of resistance of campylobacters to these agents has increased.[73] For example, a 1994 study found that most clinical isolates of *C jejuni* from US troops in Thailand were resistant to ciprofloxacin. Additionally, nearly one-third of isolates from US troops located in Hat Yai were resistant to azithromycin.[74] In another study conducted in 1997 in Minnesota, 13 (14%) of 91 chicken products purchased in grocery stores were contaminated with ciprofloxacin-resistant *C jejuni*,[75] illustrating the need for more prudent antimicrobial use in food–animal production.

Disease Caused by Clostridium botulinum

Clostridium botulinum produces botulinum toxin, which causes the clinical manifestations of botulism. Botulinum toxin, with a lethal dose of about 1 μg/kg, is the most potent of the natural toxins.[76] There are seven antigenic types of toxin, designated A through G with most human disease caused by types A, B, and E. Botulinum toxins A and B are most often associated with home canning and home-prepared foods, whereas botulinum toxin E is exclusively associated with ingestion of aquatic animals. Interestingly, the incidence of botulism in Alaska is among the highest in the world, and all cases of foodborne botulism in Alaska have been associated with eating traditional Alaska native foods, mostly from marine mammals; most of these cases were caused by toxin type E.[77] From 1990 to 2000, 160 foodborne botulism events affected 263 persons in the United States. Of these, 67 required intubation, and 11 deaths occurred.[77] Food items commonly associated with botulinum intoxication included homemade salsa and home-bottled garlic in oil.

Clinical illness is characterized by cranial nerve palsies, followed by symmetric descending flaccid muscle paralysis, which may involve the respiratory muscles. Full recovery may take weeks to months. Therapy includes intensive care support, mechanical ventilation as necessary, and timely administration of equine antitoxin.[78] See Chapter 14 for an in-depth discussion of the botulinum toxin.

Disease Caused by Escherichia coli O157:H7

Diarrheagenic *E coli* strains are important causes of diarrhea in humans. These strains have been divided into different pathotypes, according to their virulence attributes and mechanisms involved in the disease process. The major groups of intestinal pathogenic *E coli* strains include enteropathogenic *E coli* (EPEC), enteroaggregative *E coli* (EAEC), enterotoxigenic *E coli*, enteroinvasive *E coli* (EIEC), and enterohemorrhagic *E coli*.[79] Sometimes enterohemorrhagic *E coli* are known as Shiga toxin-producing *E coli* (STEC) and can be spread by food or water.

One STEC strain in particular, *E coli* O157:H7, has emerged as a cause of serious pediatric illness worldwide. Production of Shiga toxins depends on the presence of stx genes, located in the bacterial genome on lambdoid prophages, which are classified as mobile

genetic elements. It is these intrinsic Shiga toxins that can initiate a cascade of events that includes bloody diarrhea and HUS (exhibited by microangiopathic hemolytic anemia), acute renal failure, and thrombocytopenia.[80] HUS occurs in about 4% of all reported cases, and those younger than 5 years of age are at greatest risk for HUS subsequent to *E coli* O157:H7 infection.[55] The mortality rate for HUS is 3% to 5% and about 5% of the survivors have severe consequences, including end stage renal disease or permanent neurologic damage.[81] Antibiotic treatment of *E coli* O157:H7 is not recommended.[82] There is anecdotal evidence for an increase in the risk of HUS with the use of some antimicrobial agents. However, conclusive proof of this occurrence is lacking. Fluid replacement is the cornerstone of the treatment of diarrheal illness caused by enterohemorrhagic *E coli*.

The primary source of *E coli* O157:H7 is beef cattle. Current animal agricultural practices of grain (rather than hay) feeding of these animals decreases the pH in the colon, thereby promoting acid resistance in the bacteria and enhanced growth promotion for *E coli* pathogens.[83]

Disease Caused by Escherichia coli O104:H4

In May through June of 2011, two separate outbreaks of bloody diarrhea and HUS occurred in Europe. One was centered in Germany and comprised 3,816 cases of bloody diarrhea, 845 cases of HUS and 54 deaths; whereas, the other occurred in France and comprised 15 cases of bloody diarrhea, 9 of which progressed to HUS.[84,85] These cases, however, were not caused by *E coli* O157:H7. These outbreaks were caused by a more virulent form of Shiga toxin-producing *E coli* called *E coli* O104:H4 and represented the highest frequency of HUS and death recorded from an STEC strain. Epidemiological investigation determined that contaminated sprouts were the source of the outbreak and was a consequence of tainted fenugreek seeds from an exporter in Egypt that were obtained by a German seed distributor supplying a German sprout farm.[84] Tainted water may have led to contamination of sprout seeds that were exported from Egypt and distributed to farms in Europe. A portion of the original seed shipment was also sent to an English seed distributor, which repackaged the seeds and supplied them to French garden stores, leading to the outbreak in France.[86]

Disease Caused by Salmonella Species

Salmonella species infect an estimated 1.4 million persons annually in the United States. Severe infections are not uncommon, although most infections are self-limiting with diarrhea, vomiting, abdominal cramps, and fever. Estimates suggest that approximately 15,000 people are hospitalized and more than 500 deaths occur each year from *Salmonella* infections.[55] Food animals are the primary reservoir for human nontyphoidal *Salmonella* infections. Thousands of *Salmonella* serotypes exist, and many naturally inhabit the avian, mammalian, and reptilian gastrointestinal tracts. Poultry is the main source of the salmonellae in the food supply, but other vehicles for disease transmission include raw salads, milk, water, and shellfish.

Infection with many *Salmonella* serotypes cause gastroenteritis with associated diarrhea, vomiting, febrile illness, headache, and dehydration. Septicemia, enteric fever, and localized infections may also evolve from *Salmonella* infection. The most highly pathogenic of the salmonellae is *S typhi*, which causes typhoid fever, for which symptoms include septicemia, high fever, headache, and gastrointestinal illness. *S typhimurium* was the pathogen used in 1984 by an Oregon cult with intent to make people ill by deliberate contamination of salad bars.[87] More than 750 cases of illness resulted, but no deaths occurred, which may have not been the case had *S typhi* been chosen as the pathogenic biological weapon. A 1985 salmonellosis outbreak affecting more than 16,000 persons caused by cross-contamination of pasteurized with unpasteurized milk demonstrates the potential for large-scale illness caused by the salmonellae in the current food distribution system.[88]

Tickborne Diseases

Borreliosis

Lyme arthritis, as a distinct clinical entity, was recognized as early as 1972 in residents of three communities in eastern Connecticut.[89] Lyme disease or Lyme borreliosis is now the most commonly reported arthropod-borne illness in North America and Europe. In 1981, Dr Willy Burgdorfer and colleagues at the Rocky Mountain Laboratories in Hamilton, Montana, first observed spirochetes in adult deer ticks (*Ixodes scapularis*; then called *Ixodes dammini*) collected from vegetation on Shelter Island, New York, a known endemic focus of Lyme disease.[90] The bacteria were shown to react specifically with antibodies from Lyme disease patients,[90,91] and later, spirochetes were isolated from the blood of two patients with Lyme disease,[92] proving the infection's spirochetal etiology.[91] The spirochetes were later named, *Borrelia burgdorferi* (Figure 25-4), after Dr Burgdorfer. *I scapularis* (Figure 25-5) is now considered the primary vector of Lyme disease in the northeastern and north central United States. Other vectors are closely related ixodid ticks,

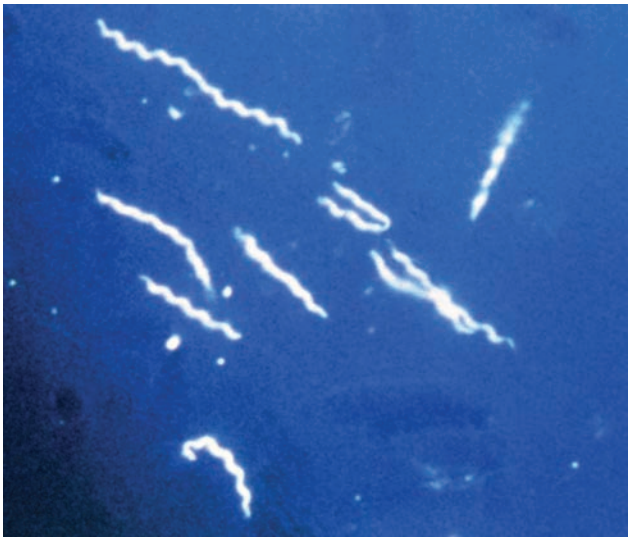


Figure 25-4. Darkfield photomicrograph of the Lyme disease spirochete, *Borrelia burgdorferi*, magnified 400x.
Photograph: Courtesy of Centers for Disease Control and Prevention Public Health Image Library.

including *I pacificus* in the western United States, *I ricinus* in Europe, and *I persulcatus* in Asia. Based on genotyping of bacterial isolates, *B burgdorferi* has now been subdivided into multiple *Borrelia* species or genospecies.[93] In North America, all strains belong to the first group, *B burgdorferi sensu stricto*. This species, along with two others, *B afzelii* and *B garinii*, are found in Europe, although most of the disease there results from the latter two species. Also, interestingly, only *B afzelii* and *B garinii* seem to be associated with the illness in Asia.[93,94] *B japonica*, which was isolated in Japan, is not known to cause human disease.[95]

Lyme disease evolves from a red macule or papule that expands annularly like a bulls-eye rash, known as erythema migrans, which may exhibit as a single lesion or as multiple lesions. However, the erythema migrans rash does not occur in all Lyme disease cases. Early systemic manifestations can include malaise, fatigue, fever, headache, stiff neck, myalgia, migratory arthralgias, and lymphadenopathy, which may last for several weeks if untreated. In weeks to months after erythema migrans onset, neurological abnormalities may develop, including facial palsy, chorea, cerebellar ataxia, motor or sensory radiculoneuritis, myelitis, and encephalitis; these symptoms fluctuate and may become chronic. Cardiac abnormalities and chronic arthritis also may result.[82]

Surveillance for Lyme disease in the United States began in 1982, and it was designated a nationally notifiable disease in 1991. Since then, the number of reported cases has increased steadily with 17,029 cases reported in 2001.[96] In 2002, 23,763 cases were reported, an increase of 40% from the previous year.[96] In 2015, approximately 300,000 people were diagnosed with Lyme disease in the United States. As with other tickborne diseases, this continued emergence of Lyme disease underscores the need for persons living in endemic areas to reduce their risk for infection through proper pest management, landscaping practices, repellent use, and prompt removal of ticks.

A newly recognized tick-transmitted disease that produces a rash (erythema migrans) similar to, and often indistinguishable from, that seen in Lyme disease has been identified in the southeastern and south central United States.[97–99] Unlike Lyme disease, however, symptoms develop following the bite of a lone star tick, *Amblyomma americanum* (Figure 25-6). The disease is named southern tick-associated rash illness,



Figure 25-5. Ixodes scapularis tick, also called the black-legged tick, is found on a wide range of hosts and is considered the main vector of the Lyme disease spirochete, *Borrelia burgdorferi*. *I scapularis* is also a vector of *Anaplasma phagocytophilum* and *Babesia microtii*, the causative agents of human granulocytic ehrlichiosis and babesiosis, respectively.
Photograph: Courtesy of James Gathany and provided by Michael L Levin, PhD, Centers for Disease Control and Prevention Public Health Image Library. Image 1669.



Figure 25-6. A female lone star tick, *Amblyomma americanum*, found throughout the southeastern United States. These ticks are considered the main vectors of *Ehrlichia chaffeensis* and *Borrelia lonestari*, the agents of human monocytotropic ehrlichiosis and southern tick-associated rash illness, respectively.
Photograph: Courtesy of James Gathany and provided by Michael L Levin, PhD, Centers for Disease Control and Prevention Public Health Image Library. Image 4407.

but it has also been referred to as Master's disease, or southern Lyme disease. *A americanum* ticks are not known to be competent vectors of *B burgdorferi*, and serological testing for Lyme disease in southern tick-associated rash illness patients are typically negative, despite microscopic evidence of spirochetes in biopsy samples. Physicians and researchers speculated that a new tick-associated spirochete may be responsible. Subsequently, molecular evidence of a novel *Borrelia* species was reported from *A americanum* ticks, from white-tailed deer, and from the skin of a patient with southern tick-associated rash illness.[100–103] The organism, named *Borrelia lonestari*, was initially described only by polymerase chain reaction (PCR) amplification of the flagellin B gene (fla B) and 16S ribosomal DNA,[104] but it has now been isolated in culture and more extensively studied.[105]

Another new tickborne *Borrelia* species has emerged to cause disease in humans. A novel *Borrelia* species was first isolated from ixodid ticks from Japan in 1995 and named *B miyamotoi*.[106] Subsequently, the bacterium was detected in ixodid ticks from North America[107,108] and Europe.[109,110] In 2011, *B miyamotoi* infection was detected in 46 patients from Russia.[111] All patients reported recent tick bite and were hospitalized with influenza-like illness with fever, headache, fatigue, myalgia, proteinuria, and elevated hepatic aminotransferase levels.[111] Cases were first described in North America in 2013.[112,113] All cases in North America have been in persons living in Lyme disease-endemic regions of the northeastern United States. Interestingly, *B miyamotoi* is genetically more similar to the tickborne relapsing-fever borreliae, which are transmitted by soft (argasid) ticks, not hard (ixodid) ticks. Some patients infected with *B miyamotoi* have even presented with clinical symptoms of relapsing fever.[111]

True relapsing fever borreliae have been known for many decades and are transmitted by ticks or lice. Relapsing fever caused by the spirochete *B recurrentis* can be transmitted by the body louse *Pediculus humanus*. *B hermsii*, which is the causative agent of tickborne relapsing fever, is transmitted by the soft tick *Ornithodoros hermsi*.[114] The disease results in fever lasting 2 to 9 days with 1 to 10 relapses. Although the total duration of louseborne disease usually averages 13 to 16 days, the tickborne disease is often longer. Gastrointestinal and respiratory involvement is common. Neuropsychiatric symptoms have also occurred.[82] Relapsing fever was first reported in the United States in 1915[115] and normally occurs in the higher elevations of the western United States and southern British Columbia (Canada). A tickborne relapsing fever outbreak occurred for the first time in Montana in 2002 among five persons visiting a cabin in the western part of the state.[114] Spirochetes were isolated from two of the patients and were identified as *B hermsii* and *O hermsi* ticks were collected from the cabin where the patients slept. This was the first report of both *B hermsii* and *O hermsi* in Montana, suggesting the risk of infection may be expanding beyond the previously recognized geographic range.

Anaplasmosis/Ehrlichiosis

Human granulocytic anaplasmosis is caused by infection with *Anaplasma phagocytophilum*, whereas the agent of human monocytotropic ehrlichiosis is *Ehrlichia chaffeensis*. Monocytotropic ehrlichiosis occurs in rural and suburban areas south of New Jersey to Kansas and in California, while granulocytic anaplasmosis occurs in areas where Lyme disease is endemic.[82] The *A americanum* tick (see Figure 25-6) transmits *E chaffeensis*, while *I scapularis* (see Figure 25-5), the Lyme disease vector, also transmits *A phagocytophilum*. A spectrum of mild-to-severe, life-threatening, or fatal disease occurs with anaplasmosis. About 20% of patients have meningoencephalitis. Infection with *A phagocytophilum*

is characterized by acute and often self-limited fever, malaise, myalgia, thrombocytopenia, leucopenia, and increased hepatic transaminases.[82] Illness ranges from mild to severe, with less than 1% case fatality.

As the *I scapularis* tick is the vector for transmission of *B burgdorferi*, *B miyamotoi*, *A phagocytophilum*, and *B microti*, coinfections of Lyme disease (and Lyme-like disease), anaplasmosis, and babesiosis (caused by the protozoan *Babesia microti*) can occur from the bite of this tick. In the United States, ticks of the *Ixodes* genus can transmit all of these diseases as well as the viral pathogens Powassan virus and the related deer–tick virus.[82,116] Coinfections with babesiosis and Lyme disease are known at times to increase the severity of both diseases.[82]

Emerging Antibiotic Resistance

Antimicrobial resistance is not a new phenomenon. Sulfonamide-resistant *Streptococcus pyogenes* emerged in military hospitals in the 1930s, and penicillin-resistant *Staphylococcus aureus* appeared in London civilian hospitals soon after the introduction of penicillin in the 1940s.[117] However, the number of resistant organisms, the geographic regions affected by drug resistance, and the number of bacterial species that are multidrug resistant (MDR) is increasing. Since the 1980s, a reemergence of tuberculosis has occurred that is often caused by MDR *Mycobacterium tuberculosis*[118] and requires the use of several—sometimes six to seven different—drugs to treat.[119] After initial reports in 2006 from South Africa of extensively drug-resistant tuberculosis (defined as tuberculosis caused by strains of *M tuberculosis* resistant to rifampicin, isoniazid, fluoroquinolones, and any of the second-line injectable drugs such as capreomycin, amikacin, and kanamycin), the number of countries reporting cases of extensively drug-resistant tuberculosis has increased to at least 84.[120] Additionally, cases of vaguely defined totally drug-resistant tuberculosis have been reported.[121,122] Other notable examples of MDR strains worldwide include *Enterococcus faecium*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *S aureus*, *Acinetobacter baumannii*, and *P aeruginosa*.[117] In developing countries, MDR enteric bacteria such as *Salmonella enteritidis*, *Shigella flexneri*, and *V cholerae* are major threats to public health.

Salmonella antibiotic resistance has emerged to become a serious concern in agriculture as well as patient management.[73,123,124] Antibiotic resistance in *E coli* O157:H7 has been shown to occur rapidly following exposure to various antibiotics, including triclosan, chloramphenicol, erythromycin, imipenem, tetracycline, and trimethoprim, as well as to a number of biocides.[125]

Few antibiotics are more potent than vancomycin. The emergence of microbial vancomycin resistance continues to be of increasing concern to clinicians and public health professionals, and surveillance systems have been instituted to monitor these pathogens.[126] *S aureus* is an important cause of illness and death and accounts for about one-fifth of bacteremia cases in the United States.[127] The discovery of vancomycin resistance in *S aureus* clinical isolates could portend the end of the antibiotic era in medicine.[42,75]

Both hospital and home healthcare patients are significantly affected by the growing emergence of antibiotic resistance.[127,128] Restrictive guidelines have therefore been developed for the use of vancomycin and other glycopeptide antimicrobials. These guidelines include a recommendation against the routine use of vancomycin as perioperative antibiotic prophylaxis for surgical site infections.[129] Vancomycin-intermediate resistance among *S aureus* has also been identified, and subsequent guidance has been developed for their identification and control of transmission.[42]

The carbapenem class of antimicrobials, which comprises imipenem, meropenem, ertapenem, and doripenem, is often the last resort for the safe and effective treatment of infections caused by MDR gram-negative bacteria, including the extended-spectrum β-lactamase (ESBL)-producing *Enterobacteriaceae*. Resistance to carbapenems occurs through several mechanisms, including the production of carbapenemases. The vast majority of acquired carbapenemases belong to one of three classes of β-lactamases, namely class B (metallo-β-lactamases) or classes A and D (serine carbapenemases). The class A group includes *Klebsiella pneumoniae carbapenemase* (KPC), which is currently the most common carbapenemase and was first detected in North Carolina in 1996 and has since spread worldwide.[130] KPC made headlines when it caused an outbreak among 18 patients at the National Institutes of Health Clinical Center in 2011.[131] Six of the patients died from their infections. The use of genomic sequencing to determine the source of this outbreak illustrates the application of this technique in epidemiological investigations (see next section).

The past few years have seen an emergence of a new type of carbapenemase, designated New Delhi metallo-β-lactamase-1 (NDM-1). It was first described in 2009 in *Klebsiella pneumoniae* (Figure 25-7) isolated from a patient receiving treatment for a urinary tract infection in a Swedish hospital, but who was of Indian origin and had previously received medical care in New Delhi, India.[132] Since this first reported case in 2009, NDM-1 producing

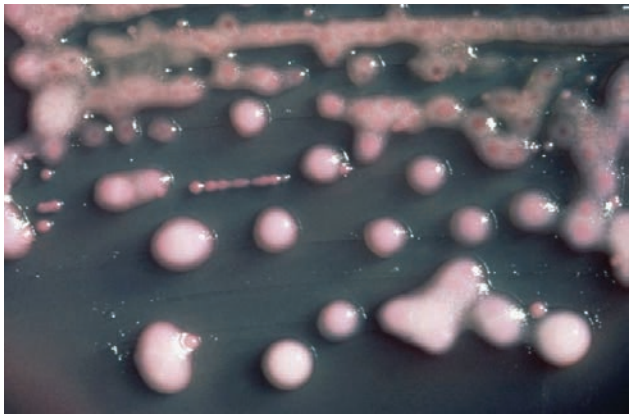


Figure 25-7. Colonies of *Klebsiella pneumoniae*, the bacterium in which New Delhi metallo-β-lactamase-1 was first identified. Magnification ×10.
Photograph: Courtesy of Centers for Disease Control and Prevention Public Health Image Library.

bacteria have rapidly spread to every continent except for Central and South America. In most of these cases, patients had been hospitalized in India, Pakistan, or Bangladesh, or had spent some time in that part of the world. It therefore suggests that the Indian subcontinent is currently the main reservoir of NDM-1 producers.[133]

A substantial number of patients have been part of the growing phenomenon of "medical tourism" resulting from delays for medical interventions such as hip and knee replacements, spinal surgery, and ophthalmologic procedures. It is estimated that in 2012 as many as 1.6 million Americans received healthcare outside of the United States.[134] Many of these medical tourists undergo such procedures in India, which may put them at risk of contracting NDM-1 strains of bacteria. NDM-1 has been identified mostly in *E coli* and *K pneumoniae*, in many cases in strains that are already MDR, making these bacterial pathogens resistant to virtually every clinically available antibiotic.

This is even more alarming considering the decreasing number of potentially new antibiotics that have come through the pharmaceutical pipeline in recent decades. The reasons for this decline are many and diverse.[135] Some of these reasons include the nature of antibiotic use, which is typically short term, compared to other drugs; the drug's uncertain future because of the constantly evolving nature of antibiotic resistance; and governmental over-regulation. Thus, appropriate antibiotic use will continue to be an important issue for clinicians and epidemiologists for the foreseeable future.[136]

Genomic Epidemiology—Use of Whole Genome Sequencing to Track Epidemics of Bacterial Pathogens

Historically, public health investigators have used techniques such as DNA–DNA hybridization, patterns of restriction endonuclease digestion of DNA on agarose gels, or pulsed-field gel electrophoresis, to determine the relatedness of bacterial pathogens isolated from different patients or different geographic regions. Although these methods can provide some information regarding strain relatedness, they vary greatly in their resolution. Bacterial genotyping techniques commonly used in outbreak investigations have limited power of resolution because they target only small parts of the genome. More recently, whole genome sequencing has emerged as a rapid and high-resolution method to investigate bacterial disease outbreaks[137–139]; this application of the technique is often referred to as "genomic epidemiology."[140] Two recent high-profile examples of using this technology to track the origin and transmission of bacterial pathogens during outbreaks include the 2010 cholera epidemic in Haiti[24] and the 2011 German *E coli* O104:H4 outbreak[84] discussed previously.

Cholera had not been previously reported from Haiti; thus, the main question was from where did the strain of *V cholerae* responsible for the outbreak come? The source of the cholera in Haiti has been controversial, with three main hypotheses being suggested. The first hypothesis was that the pathogen arrived to Haiti from the Gulf of Mexico because of tectonic shifts resulting from the earthquake. The second hypothesis was that the pathogen evolved into disease-causing strains from nonpathogenic strains naturally present in Haiti. The third hypothesis was that the pathogen was somehow inadvertently introduced into the Haitian environment, triggering the epidemic.[24] A specific form of this hypothesis, that Nepalese soldiers from a United Nations military camp were the direct source of the cholera, was a commonly held belief in Haiti. To resolve this question, Matthew Waldor of Harvard Medical School collected several samples of the *V cholerae* strain circulating in Haiti and sent them to colleagues at Pacific Biosciences, a biotech company that manufactures third-generation single-molecule real-time DNA sequencers. Scientists at Pacific Biosciences sequenced DNA from two samples from the Haitian outbreak, one strain that caused cholera in Latin America in 1991, and two *V cholerae* clinical strains isolated from Bangladesh in 2002 and 2008 and compared them to reference genomes already in the database. They analyzed single nucleotide and copy

number variations to determine the likely phylogeny of the Haitian strain and found that the Haitian isolates were more closely related to the strains from Bangladesh (South Asia) and more distantly related to isolates circulating in South America. Their conclusions were that the Haitian epidemic was probably the result of the introduction of a *V cholerae* strain from a distant geographic source.[28]

Unfortunately, this determination was as specific as these investigators could get without having a more extensive collection of strains to compare. Using a different next-generation DNA sequencer (Genome AnalyzerIIx, Illumina, San Diego, CA), Frank M Aarestrup and his team at the Technical University of Denmark sequenced 24 *V cholerae* isolates collected from August to November 2010 from five different districts in Nepal. Phylogenetic analysis showed that all 24 *V cholerae* isolates from Nepal belonged to a single well-supported clade that also contained isolates from Bangladesh and Haiti. Furthermore, direct comparison between the three Haiti outbreak strains and the three most closely related strains from Nepal show a near perfect match.[29] This finding, along with epidemiological data, strongly supports the hypothesis that the *V cholerae* strains responsible for the 2010 Haitian cholera epidemic were brought to Haiti from Nepal, most likely via Nepalese soldiers serving as United Nations peacekeepers.

A similar approach (ie, rapid whole genome sequencing) was taken to fully characterize the *E coli* from the 2011 German outbreak in near real-time.[141] This comprehensive analysis took place in the first days and weeks of the outbreak, rapidly enough to inform physicians treating infected patients and epidemiologists tracing the source of the pathogen. Only this kind of rapid whole genome sequencing allowed investigators to determine that the outbreak strain was an extremely rare form of bacterium that was a "hybrid" of enteroaggregative *E coli* and enterohemmorhagic *E coli*. Researchers also determined that this was distinct from other *E coli* O104:H4 strains because it contained a prophage encoding a Shiga toxin and a distinct set of other virulence and antibiotic-resistance factors.[141]

These are only two examples of the use of genomic epidemiology. However, it is clear that as the speed and accuracy of next-generation DNA sequencing increases (and the cost decreases), it is likely that in the near future, it will be as common a diagnostic tool as the PCR is today.

EMERGING VIRAL DISEASES

Avian Influenza and the Threat of Pandemics

Influenza is a highly contagious, acute respiratory illness, with clear evidence of human infections dating back to the Middle Ages, and probably occurring as far back as ancient Greece and Rome. The influenza viruses are members of the *Orthomyxoviridae* family and contain a segmented negative-sense RNA genome.[142] There are three genera of influenza viruses: influenza A, B, and C. Influenza A and B viruses are associated with seasonal epidemic illness in humans; whereas, influenza C infections in humans are sporadic. Because influenza A viruses are the only type of influenza viruses that have caused pandemics in the human population, this section will focus on this influenza virus type. The genome of influenza A viruses comprises eight gene segments, encoding 10 to 12 proteins.[142,143] The segmented nature of the genome allows for reassortment, or the exchange of segments (and genes) between two or more virus strains co-infecting the same cell. The major surface glycoproteins of influenza A viruses, hemagglutinin (HA) and neuraminidase (NA), are the major antigens of the virus. HA and NA are involved in the interactions between the virus and host cells, and they are the major targets of neutralizing antibodies. These proteins are seen as spikes in electron micrographs (Figure 25-8). The HA binds to sialic acid-containing moieties on the cell surface, mediating attachment and entry of the virus, and the NA is a receptor-destroying enzyme that cleaves sialic acids from the glycan backbone, thus facilitating release and spread of the virus. Subtypes of influenza A viruses are designated by their particular HA and NA types (to date, distinct subtypes of influenza B and C viruses have not been observed). Sixteen HA and 9 NA subtypes have been identified in aquatic birds, which act as the major reservoir for influenza A viruses in nature. In addition, influenza A viruses can infect many mammalian species, including pigs, horses, dogs, cats, ferrets, mink, whales, and seals. Influenza A viruses of the most recently described subtypes, H17N10 and H18N11, have not been isolated; partial genome sequences of these highly divergent influenza A viruses were identified in bats from Guatemala and Peru (see section on Influenza Viruses in Bats).[144,145] Thus far, only influenza A viruses carrying one of three HA subtypes (H1, H2, H3) have been able to achieve sustained transmission and establish themselves in the human population, causing subsequent seasonal

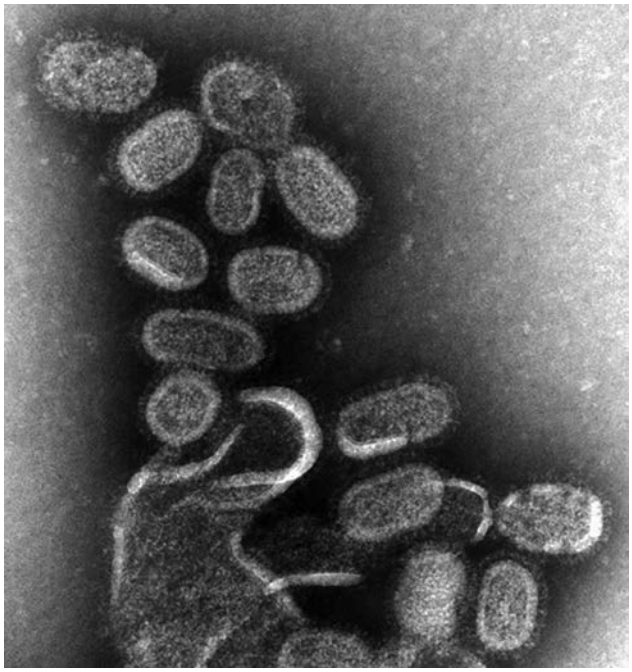


Figure 25-8. Negative-stained transmission electron micrograph showing the reconstructed 1918 influenza virons collected from the supernatants of virus-infected Madin-Darby Canine Kidney cell culture 18-hours postinfection. Surface spikes (hemagglutinin and neuraminidase) can be clearly seen extending from the surface of the virons.
Photograph: Courtesy of Cynthia Goldsmith and provided by Dr Terrence Tumpey, Centers for Disease Control and Prevention Public Health Image Library. Image 8160.

epidemics. For example, one circulating influenza virus strain is designated subtype H3N2 and has been the most commonly isolated strain during the last 4 decades.

Antigenic diversity in influenza A viruses can result from changes in the HA and NA genes. One type of variation called "antigenic drift" occurs as a result of accumulation of point mutations in the genes encoding HA and NA proteins. These point mutations occur randomly as the virus is copied in infected cells and are largely responsible for the annual epidemics of influenza seen during the winter months, and for the frequent need to reformulate the seasonal influenza vaccine.

Another type of change that can occur is called "antigenic shift," which results from the reassortment of genes that occurs when two different influenza viruses infect the same host cell, causing a shift in the HA and/or NA type of the virus. This phenomenon results in the emergence of novel influenza A strains that have the potential to cause widespread infection and disease in a susceptible population. Since 1933, when the influenza A virus was first isolated (an H1N1 subtype), major antigenic shifts (and pandemics) have occurred in 1957 ("Asian influenza," an H2N2 subtype virus) and in 1968 ("Hong Kong influenza," an H3N2 subtype virus). In 1977, the H1N1 subtype virus reappeared after a more than 20-year hiatus; however, this time it did not cause severe disease, most likely because of the immunity of persons older than 20 years of age who had been infected with the virus when it circulated earlier in the century. It is highly unlikely that this virus was maintained in an animal host for more than 20 years without changes. One possible explanation is that the virus was maintained in a laboratory freezer until it somehow was reintroduced into the human population.

In 2009, a swine-origin H1N1 influenza A virus caused the first influenza pandemic of the 21st century (see section on Swine Influenza and the H1N1 Influenza Pandemic, 2009). This H1N1 influenza A virus then replaced the circulating seasonal H1N1 influenza viruses, and continues to cause seasonal epidemic infections, with typically mild-to-moderate illness.

Of the three influenza pandemics that occurred in the 20th century, the pandemic of 1918 to 1919 was the most devastating, causing an estimated 20 to 40 million deaths worldwide. Unusually, young healthy adults between 20 and 40 years of age accounted for almost half of the influenza deaths during this pandemic. The epidemic spread rapidly, moving around the globe in less than 6 months. It is estimated that the pandemic killed 675,000 Americans, including 43,000 servicemen who were mobilized for World War I (Figures 25-9 and 25-10), and it may have played a significant role in ending the war.[146] Its impact was so profound that the average life expectancy in the United States temporarily declined by more than 10 years.[147]

Analysis of survivor antibody titers from the late 1930s suggested that the 1918 strain was an H1N1 subtype virus closely related to classical swine influenza viruses.[148] Researchers at the Armed Forces Institute of Pathology in Washington, DC—who isolated influenza viral RNA from preserved lung tissue of US servicemen who died during the 1918 pandemic, and also from a victim of the pandemic who was buried in a mass grave in Brevig Mission, Alaska—ultimately confirmed this theory. Over the next decade, all eight gene segments of the 1918 influenza virus were reconstructed, sequenced, and characterized.[149] Unfortunately, no obvious genetic changes were observed in any of these gene sequences that would account for the exceptional virulence of this pandemic virus.[150] The reconstructed virus was highly virulent in animal models, including



Figure 25-9. Emergency hospital during the 1918 influenza pandemic, Camp Fuston, Kansas. NCP 1603.
Photograph: Courtesy of the Otis Historical Archives, National Museum of Health and Medicine, Washington, DC.

mice, ferrets, and nonhuman primates.[151,152] It was determined that the HA and polymerase complex genes played important roles in virulence, although no single property of the virus has been identified to fully explain the devastating mortality seen in 1918 and 1919.[143]

Host factors undoubtedly played some role. It has been suggested that an uncontrolled cytokine response was elicited by the virus, leading to immunopathology. However, most of the mortality during the pandemic appeared to be attributable to secondary bacterial pneumonia.[9] Viral and host factors could be involved in increased susceptibility to bacterial infections, and this remains an active area of investigation. In addition, the fact that the pandemic occurred in the preantibiotic era also likely contributed to the high mortality observed.

Much has been learned from the remarkable achievement of the resurrection of the 1918 virus. Continued study of this pathogen will continue to provide valuable information for the development of vaccines and treatments for future pandemic influenza viruses.

Countermeasures do exist for the treatment and prevention of influenza. Annual vaccines include two influenza A strains (H1N1 and H3N2) and two influenza B strains (one from each of the two influenza B lineages that circulate in humans). The component strains are selected based on surveillance of the strains that are circulating in humans about 6 months prior to when vaccine will be needed for immunizations before the start of the influenza season. In some cases, the vaccine does not match the circulating strain, and low vaccine effectiveness is then observed. The strains may need to be updated annually due to antigenic drift. Influenza vaccines against potentially pandemic influenza viruses (eg, H5 and H7 subtypes) have been manufactured and evaluated in clinical trials, but these vaccines have been found to be suboptimally immunogenic, requiring higher doses or adjuvants to achieve the antibody responses needed for protection.[153] In recent years, there has been much interest in developing a universal vaccine that would provide broad cross-protection against multiple subtypes of influenza, including pandemic strains that may emerge in the future.[154] The identification of a highly conserved region of the influenza HA (the stem or stalk region) against which broadly cross-neutralizing antibodies have been detected in humans has spurred a great effort to develop ways of using the conserved HA stem region as an immunogen.[155–157] However, the availability of such a vaccine—if this strategy is successful—is still years in the future.

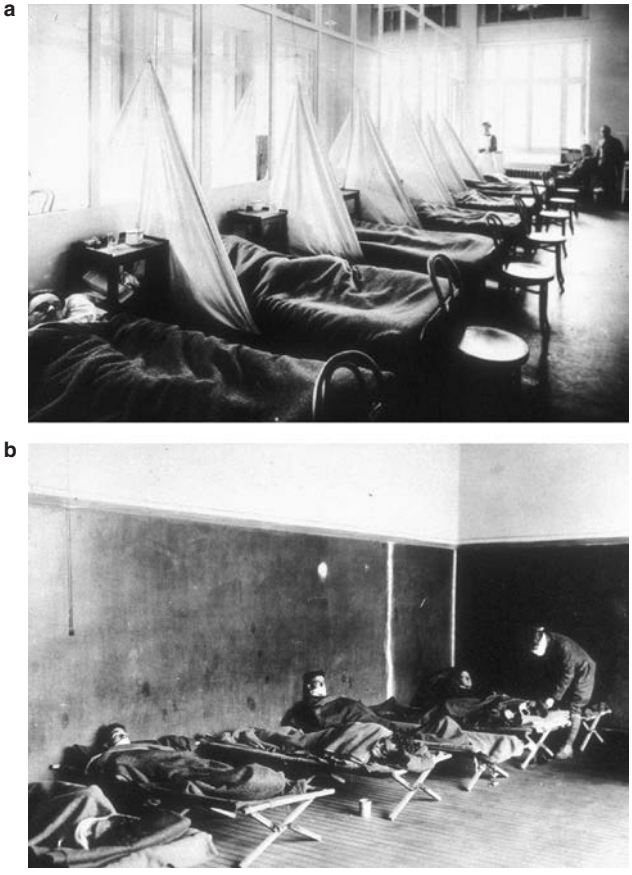


Figure 25-10. Influenza wards, US Army camp hospitals at (**a**) Aix-Les-Bains, France (Reeve 14682), and (**b**) Hollerich, Luxembourg (Reeve 15183).
Photographs: Courtesy of the Otis Historical Archives, National Museum of Health and Medicine, Washington, DC.

Two classes of drugs are available to treat influenza: (1) the NA inhibitors (oseltamivir, zanamivir) and (2) the M2 ion channel inhibitors (adamantanes). As with many antivirals, development of resistant strains is a problem that limits their use, and successful treatment with NA inhibitors must be initiated early after symptom onset to be effective.

Swine Influenza and the H1N1 Influenza Pandemic, 2009

Influenza infections in pigs were first recognized clinically during the 1918 Spanish influenza pandemic, and the first isolation of an influenza virus was from pigs in 1930.[158] Transmission of influenza virus to humans from swine has since been documented on several occasions.[159,160] Before 2009, infections of humans with swine influenza viruses were sporadic and did not result in large outbreaks of illness. In all cases, illness was indistinguishable from typical influenza virus infection in humans. Between 1958 and 2005, 37 cases of swine influenza virus infections in humans were reported, 19 of which were in the United States (reviewed in Meyers et al[159]). In 22 (61%) of these cases, recent exposure to swine was reported, and 13 of these cases resulted from occupational exposure. In the United States in 1976, there was an outbreak of swine influenza at Fort Dix in New Jersey.[161] Infection with an H1N1 swine influenza virus resulted in one soldier's death and respiratory illness in 12 additional soldiers. No exposures to pigs were reported. It was subsequently found by serological analysis that as many as 230 soldiers were infected.[161]

Influenza A viruses of the H1, H2, and H3 subtype are all present in swine. The first influenza pandemic of the 21st century occurred in 2009, and was caused by an H1N1 virus that originated in swine. The emergence of a pandemic virus from the swine reservoir was unanticipated, particularly with many influenza researchers focusing their efforts in recent years on avian influenza (AI) viruses, particularly H5N1 (see section on Human Infections with Highly Pathogenic H5N1 Avian Influenza Viruses), as possible agents for the next pandemic.

Influenza-like illness was reported in two children in southern California in March 2009, and the number of pneumonia cases increased in Mexico City around the same time. A novel H1N1 influenza A virus was isolated from individuals in the United States in April 2009. The virus was soon characterized as a quadruple reassortant virus of the H1N1 subtype, with gene segments from swine and avian influenza viruses.[162] The novel virus spread rapidly throughout the world, and the WHO declared a pandemic on June 11, 2009. Although illness caused by the nascent pandemic virus was generally mild to moderate, severe illness was observed in individuals with underlying conditions such as obesity and diabetes, in pregnant women, and—surprisingly—in older children and young adults.[163,164] This is in contrast to seasonal influenza epidemics, where the burden of disease is usually greatest in the very young and the elderly.

Antigenic characterization of the 2009 pandemic H1N1 virus and serological studies revealed that the HA was related to that of H1N1 influenza viruses that circulated in the 1930s, 1940s, and earlier, including the 1918 H1N1 influenza virus.[9,165] In addition, the 2009 pandemic H1N1 virus was antigenically similar to the H1N1 virus that caused the swine influenza at Fort Dix that triggered a national vaccination campaign in 1977.[161] Vaccination in 1977 likely afforded some protection against the pandemic virus.

The 2009 pandemic H1N1 viruses were found to be sensitive to NA inhibitors and were resistant to ion channel inhibitors. Resistance to adamantanes was conferred by a mutation in the M2 gene of the 2009 H1N1 viruses. An immediate response to the emergence of the 2009 H1N1 virus was the production of a vaccine. Vaccine manufacturers in the Northern Hemisphere had recently finished distribution of the trivalent vaccine for the 2008 to 2009 winter influenza season when the pandemic H1N1 virus emerged. Delays occurred in vaccine production, resulting from the difficulty in generating reassortant seed viruses for vaccine manufacture and the instability of the pH1N1 HA protein used for potency testing for vaccine lot release. As a result of these delays, vaccine was not distributed until the peak of infections had passed. Human clinical testing of the monovalent inactivated H1N1 vaccine revealed a high titer antibody response to vaccination in most age groups tested, providing more evidence of preexisting immunity to the pandemic H1N1 virus.[166,167]

The WHO declared the end of the 2009 influenza pandemic in August 2010. The WHO reported 18,631 laboratory-confirmed deaths caused by the H1N1 virus between April 2009 and August 2010. However, recent estimates of global mortality from this pandemic are reported to be approximately 10- to 15-fold higher.[168,169]

In 2010, the pandemic H1N1 virus replaced the circulating seasonal H1N1 influenza viruses, and it has continued to circulate concomitantly with H3N2 human influenza viruses, causing mild to moderate disease. Since the winter of 2010–2011, the pandemic H1N1 virus has been a component of the seasonal influenza vaccine. The HA of the H1N1 virus does not appear to have undergone significant antigenic drift,

and so the original vaccine strain, A/California/7/2009, has not changed (http://www.who.int/influenza/vaccines/virus/recommendations/en/).

The first influenza pandemic of the 21st century was the first influenza pandemic to occur in the molecular biology era. Much information about this virus will continue to be generated. Although delays occurred in vaccine production and deployment, the novel virus was rapidly identified and characterized. Key questions about this virus remain, including the precise point of origin of the virus and the reason for the severity of disease in pregnant women infected with this virus. These questions and other features of the virus are the subjects of intensive study.

Fortunately, the morbidity and mortality from the 2009 influenza pandemic were not on the scale of the 1918, 1957 or 1968 pandemics. The 2009 H1N1 influenza pandemic underscored several important aspects of influenza biology:

- the unpredictability of the emergence of novel influenza viruses from an animal reservoir;
- the diversity of reassortant influenza viruses in nature derived from a variety of animal hosts;
- the importance of preexisting immunity in the human population;
- the rapidity with which a human-adapted virus can spread globally; and
- the importance of surveillance of swine influenza viruses, as well as their avian counterparts.

Swine influenza viruses continue to present a pandemic threat. In 2011, and particularly in the summer of 2012, a number of cases of human infection with quadruple reassortant swine H3N2 viruses were reported. These viruses are genetically identical to the pandemic H1N1 viruses, except that the HA and NA genes are derived from circulating swine H3N2 triple reassortant viruses.[170] Importantly, the M gene segment is derived from the Eurasian swine lineage, perhaps increasing the likelihood of sustained transmission of these viruses in humans. Influenza viruses that circulate in swine are referred to as "variant" viruses when isolated in humans, so that the human cases are considered to be infections with the H3N2v virus.

From July to September 2012, 306 cases of human infection with H3N2v influenza viruses were reported.[171] H3N2v has been associated with typical influenza illness, and 16 H3N2v-associated hospitalizations and one death occurred. Almost all cases have documented histories of swine exposure, and the majority of cases were associated with attendance at state fairs. However, some cases have suggested the presence of limited person-to-person transmission.

Influenza viruses in swine do not appear to be subjected to the same immunologic pressure that leads to antigenic drift in human influenza viruses. Once introduced into swine populations, influenza viruses therefore tend to be antigenically stable. The H3N2v viruses isolated from humans are phylogenetically most closely related to human influenza viruses from the mid 1990s.[170,172] Clinical cases of H3N2v have occurred primarily in children 12 and younger, ie, individuals born after these viruses last circulated in humans.

Several studies have assessed the degree of baseline population immunity to H3N2v viruses by measuring antibody against these viruses using serum samples from different age groups. These studies have also suggested that children younger than 10 would be largely susceptible to infection based on lack of preexisting antibody.[171,172] Current seasonal inactivated influenza vaccine does not induce an antibody that recognizes H3N2v in children, although some cross-reactive antibodies are observed in adults.

These observations suggest that H3N2v viruses pose a potential pandemic risk. The viruses are prevalent in domestic swine and have a demonstrated ability to infect humans. They possess genotypes that have features that potentially enable human transmission, and some cases of human-to-human transmission have been observed. Previous swine origin viruses have already caused pandemics, and influenza viruses of the H3 subtype are clearly capable of causing widespread human disease. Although the pattern of baseline antibody possibly suggests that the impact of an H3N2v pandemic would be focused on young children, the majority of adults would also be predicted to be susceptible. Thus, development of effective vaccines for H3N2v candidate viruses is a high priority.

Human Infections With Avian Influenza Viruses

Wild aquatic birds are the major reservoirs of all subtypes of influenza A virus that have been isolated, and the viruses do not cause symptomatic infections in these species. It was generally accepted—until recently—that for an influenza pandemic to occur, AI viruses would reassort with human influenza viruses in an intermediate host, and a novel strain capable of infecting humans (with no preexisting immunity to the new virus) would emerge. Rare transmission events directly from birds or transmission of AI from other animals to humans have been reported.[173] Transmission of AI from birds to humans before 1997 occurred with AI viruses mainly of the H7 subtype.[174] Human

infections with other AI subtypes have since been reported: for example, in 1999 and 2003 with H9N2 viruses[175,176]; in 2003 with H7N7 viruses[177]; from 1997 to present with H5N1 viruses[178]; in Egypt with H10N7 viruses,[175] and in 2013 in Taiwan with H6N1.[179] These cases confirmed that AI viruses are capable of directly infecting humans without the requirement for reassortment in an intermediate mammalian host.

Human infections with AI viruses have resulted in a wide spectrum of disease, ranging from mild febrile and respiratory illness in some H5 and H9N2 influenza infections, conjunctivitis in the case of H7 influenza infections, to severe disease and death, as seen with the highly pathogenic avian influenza (HPAI) H5N1 cases that have occurred between 1997 and the present.[178] In contrast to the rarity of the isolation of AI viruses from humans, serosurveys of farmers in rural southern China suggest that many other subtypes of AI viruses have crossed the species barrier and infected humans. Specifically, seroprevalence levels of 2% to 7% for H5 viruses alone were reported,[180] and the seropositivity of human sera for H7, H10, and H11 viruses was estimated to be as high as 38%, 17%, and 15%, respectively. The dogma had been that because of receptor specificity, AI viruses were incapable of efficiently infecting humans. It has long been believed that this host restriction of AI viruses prevents the emergence of new pandemic strains via direct avian-to-human transmission. However, human cases of direct infection by AI viruses are becoming increasingly frequent; it is now known that the potential of an AI virus to infect humans is polygenic in nature, and it is widely accepted that this is not solely attributable to its HA receptor specificity. The most significant zoonotic transmissions of AI viruses in recent years have been caused by H5N1 and H7N9 AI viruses. These outbreaks are described in more detail in the following sections.

Human Infections With Highly Pathogenic H5N1 Avian Influenza Viruses

The first reported cases of H5N1 influenza infections in humans occurred in 1997 in Hong Kong. The first case was a 3-year-old boy.[181] The child died 12 days after the onset of symptoms with several complications, including respiratory failure, renal failure, and disseminated intravascular coagulopathy. An H5N1 AI virus was isolated from a tracheal aspirate specimen obtained on day 10 of illness. The nucleotide sequence of the isolate revealed a multibasic amino acid sequence at the HA cleavage site, a motif characteristic of HPAI viruses,[182] which—until this point—had only been known to cause severe disease in poultry. Phylogenetic analysis of the H5N1 Hong Kong isolate revealed no evidence of genetic reassortment with recent human influenza A viruses. The isolate was highly pathogenic for chickens and the virus displayed an AI virus-like receptor specificity. No clear epidemiological link was established between the infected child and infected poultry. However, outbreaks of influenza occurred in poultry on farms in Hong Kong between late March and early May 1997, and two viruses from one of these outbreaks were identified as H5N1 influenza viruses. It was reported that sick chickens were at the preschool attended by the child, although no evidence indicates that these chickens were infected with AI or that the child was in close contact with them.

Additional cases of H5N1 in humans in Hong Kong were confirmed in 1997.[183] In total, 18 Hong Kong residents became infected with HPAI H5N1 influenza in 1997, of whom 6 died. The cases, which were not geographically related or confined to a specific age group, occurred in children and adults with ages ranging from 1 to 60 years. In 7 of the 18 cases, histories of possible exposure to poultry existed, where the patients had either bought chickens before they became ill or had worked in proximity to chicken stalls near their homes.[184] Seven of the patients had severe complications, most prominently pneumonia, gastrointestinal manifestations, elevated liver enzyme levels, and renal failure. With one exception, patients younger than age 13 recovered from their illness whereas older patients had more severe disease that resulted in death in five cases.

An epidemiological study of the human H5N1 cases in Hong Kong in 1997 suggested that the viruses were transmitted directly from birds to humans, and serological evidence of human-to-human transmission was limited.[184] In most cases, infection was associated with recent exposure to live poultry. Sequence analysis of AI viruses circulating in China around that time resulted in the hypothesis that the H5N1 influenza viruses that infected humans in 1997 arose by reassortment between an H5N1 influenza A/goose/Guangdong/1/96-like virus and an H9N2 or H6N1 virus similar to those circulating in the live bird markets of Hong Kong in 1997. However, the actual sequence of reassortment events cannot be definitively determined from the small number of viruses available for analysis from preceding years.[173]

The human cases of HPAI H5N1 infection that occurred in 1997 coincided with further outbreaks of highly pathogenic H5N1 influenza in poultry on farms and in live bird markets in Hong Kong. Slaughter of the 1.5 million poultry in the Hong Kong Special Administrative Region was conducted between December 29 and December 31, 1997. Many experts believe that because of this action, an influenza pandemic caused

by the H5N1 virus was averted, although it has since become apparent that the H5N1 AI viruses have not adapted for efficient transmission in humans. Reintroduction of poultry to the Hong Kong Special Administrative Region began in February 1998. At this time, new practices were introduced for the live bird markets in Hong Kong. Waterfowl (eg, ducks and geese) are now sold at separate markets from chickens; ducks and geese are now slaughtered at the markets; and markets have a monthly rest day when they close for thorough cleaning, the remaining birds are culled, and restocked with fresh imported poultry. Surveillance of birds in the markets has continued since the 1997 outbreaks.

In 2003, again in Hong Kong, two cases of HPAI H5N1 infection were confirmed in a father and son of a family who had recently visited mainland China.[8] HPAI H5N1 AI infections again appeared in the human population in 2004 in Vietnam and Thailand, and they were confirmed in Cambodia, Indonesia, and China in 2005. HPAI viruses of the H5N1 subtype in Asia continued to evolve and spread in avian populations, and human cases were eventually reported in the Middle East, Europe, and Africa. Multiple genotypes and several clades of H5N1 influenza viruses have been identified.[96,185] It is believed that the highly pathogenic H5N1 viruses in Asia originated from viruses in ducks in southern China. HPAI H5N1 viruses have been isolated from dead migratory birds in Hong Kong and parts of China,[186] implicating wild birds in their spread across Asia and other parts of the world.

Human cases and fatalities have since been reported in 15 countries. As of October 2013, 641 laboratory-confirmed cases and 370 deaths have been reported to the WHO.[178] The WHO's website contains a comprehensive timeline that chronicles the panzootic spread of HPAI H5N1 viruses since 1997 and the associated incursions of the virus into the human population.[178] The HPAI H5N1 viruses of the A/goose/Guangdong lineage continue to evolve by antigenic drift, resulting in efforts to continually update stockpiled vaccine strains for use should these viruses gain the ability to transmit efficiently and cause widespread infections. Of particular concern are the HPAI H5N1 viruses circulating in Egypt since 2009, because these isolates display increased affinity for human-type sialic acid receptors.[187,188] Fortunately, HPAI H5N1 viruses have not acquired the ability to transmit efficiently from person to person, although several small family clusters of cases have been reported.[189,190]

Despite the global prevalence of HPAI H5N1 infections in birds, and the number of reported infections in humans, these viruses have not yet acquired the necessary genetic changes required for efficient, sustained transmission in the largely immunosusceptible human population. Recent controversial studies involving intentional introduction of mutations into HPAI H5N1 viruses to confer efficient transmissibility in the ferret model—the preferred animal model for the study of influenza transmission—resulted in the identification of several changes in the viral genome that achieved this state.[188,191] Changes in the HA gene and the PB2 polymerase gene were found to be necessary—but not sufficient—to confer transmissibility in ferrets.[192]

Human Infections With H7N9 Avian Influenza Viruses, 2013

Human cases of H7 AI have typically been associated with large outbreaks of H7 AI infection in birds, caused by either highly pathogenic or low pathogenicity viruses. With the exception of a fatal infection by a HPAI H7N7 in the Netherlands in 2003,[177] illness associated with H7 AI infections in humans has been relatively mild.

Recent human cases of H7N9 AI infection in China have caused great concern regarding the potential emergence of an influenza pandemic. Human infections with H7N9 AI viruses were first reported in China on March 31, 2013.[193] The first three cases reported were in two individuals from Shanghai and one individual from Anhui.[194] All three patients died. Between April and the end of May 2013, 132 laboratory confirmed cases had been reported to the WHO, 37 of them fatal. Cases occurred in eight contiguous provinces of eastern China and in the two municipalities of Beijing and Shanghai, and a single case was reported in Taiwan.[195] The infected individual from Taiwan had recently travelled to Jiangsu Province in China.

In the majority of the laboratory-confirmed cases of H7N9 in China and in the case reported in Taiwan, illness was severe.[194,195] In the initial three fatal cases reported in China, all three patients presented with fever, cough, and dyspnea. Radiologic findings were consistent with pneumonia, with diffuse opacities and consolidation.[194] The patients progressed rapidly to acute respiratory distress syndrome (ARDS) and multiorgan failure. Gao et al[194] reported the clinical features of an additional 111 laboratory confirmed cases in China. The most common early symptoms were fever and cough. Ninety-seven percent of these patients had findings consistent with pneumonia upon admission to the hospital, 77% were admitted to the intensive care unit, and 27% of patients died. The median age of patients was 61 years; 68% were male and 61% had at least one underlying medical condition—most commonly, coronary heart disease, hypertension, diabetes, or chronic obstructive pulmonary disease. Presence of an underlying medical condition

was identified in this study as the only independent risk factor for progression to ARDS. The most common complications of H7N9 AI infection in these patients were ARDS (71%), shock (26%), acute kidney injury (16%), and rhabdomyolysis (10%). Pneumonia and ARDS occurred in all fatal cases; progression to severe pneumonia, ARDS, and shock was rapid. The leading cause of death was refractory hypoxemia. In all cases, patients were treated with neuraminidase inhibitors.

It has been suggested that many mild cases may have occurred but were not reported.[179] Nevertheless, it is clear that the avian-origin H7N9 virus is capable of causing severe disease and death in humans, and age and underlying medical conditions are risk factors for severe disease.

The possible origin of the H7N9 AI viruses that emerged in China in 2013 has been extensively studied. Phylogenetic analyses determined that the viruses are the result of multiple reassortment events, and they have gene segments related to those from at least three different types of AI viruses (Figure 25-11).[194,196] The HA genes are most closely related to those from low pathogenicity AI H7N3 viruses that were isolated in parts of Asia, including China, in 2011 (A/duck/Zhejiang/12/2011-like). The NA genes are most closely related to those from low pathogenicity AI H7N9 viruses that circulated in South Korea in 2011 (A/wild bird/Korea/A14/2011-like); although notably, the human H7N9 viruses have a 15-amino acid deletion in the NA stalk region that had previously been reported to be associated with the adaptation of AI viruses to terrestrial poultry.[194,196,197] The donors of the six internal protein genes were H9N2 viruses.[194,198] It appears that ducks and chickens were probably intermediate hosts for the H7N9 reassortant viruses.

The H7N9 AI viruses isolated from humans and from birds at the time of the outbreak lack the multibasic amino acid cleavage motif in the HA gene that is seen in HPAI viruses. In addition, infection of avian species with H7N9 isolates did not result in disease, although virus was shed and the birds developed antibodies. These observations underscore the challenge for surveillance of these viruses in avian populations, since they do not cause overt disease.[193] The source of the H7N9 AI viruses appears to be live bird markets.[199,200] H7N9 AI viruses isolated from live bird markets were almost identical in sequence to the human isolates. However, H7N9 virus was isolated from only a small percentage of samples taken from birds and the environment all across China. All samples taken from swine and from slaughterhouses were negative for H7N9 AI.

In summary, AI viruses of the H7 subtype have been directly transmitted to humans on numerous occasions. The recent emergence of H7N9 AI infections in humans in China confirmed that H7 AI viruses have the potential to cause severe disease. Studies have demonstrated that the H7N9 AI viruses isolated from human cases bear some genetic markers that are associated with adaptation to mammals,[196,198] and their ability to transmit efficiently in the ferret model by direct contact and by respiratory droplets has been demonstrated.[201,202] Characterization of the crystal structure of the H7N9 AI HA glycoprotein showed preferential recognition of avian-like receptors by H7N9 AI viruses isolated from humans, suggesting that the virus may be poorly adapted for mammalian transmission at this time.[179] Although sustained, efficient human-to-human transmission has not been observed, the pandemic potential of these viruses is of great concern. It is expected that more human cases will occur in the winter months. In December 2013, two human cases of H7N9 AI infection in Hong Kong were reported. In both cases, the infections were thought to have been acquired in the neighboring city of Shenzhen (http://www.chp.gov.hk/en/view_content/32486.html). As of January 31, 2015, 677 laboratory-confirmed cases of human infection with H7N9 AI had been reported to the WHO, with at least 275 deaths.[203]

Influenza Viruses in Bats

In recent years, an increasing interest has emerged in the role of bats as reservoirs of viral pathogens.[204] The coronavirus that caused the SARS outbreak in 2003 (SARS-CoV) is closely related to CoV genomic sequences found in bats in China (see section on Diseases Caused by Emerging Coronaviruses). The paramyxoviruses Hendra and Nipah were isolated from bats. Bats are thought to be possible reservoirs of filoviruses, including Ebola virus and Lloviu virus, a novel filovirus[205,206] since viral nucleic acid sequences have been identified in a variety of bat species. Additionally, Marburg virus has been isolated from Egyptian fruit bats (*Rousettus aegyptiacus*).[207–209]

In 2012, Tong and colleagues[144] reported the identification of novel influenza virus sequences in little yellow-shouldered bats in two locations in Guatemala. Virus was not isolated, but nucleic acid sequences were derived from rectal swab samples, and from liver, intestine, and kidney tissue samples. The sequences were identified as originating from a highly divergent influenza virus. The novel virus was designated as belonging to a new subtype of influenza A viruses, H17N10. The NA gene was the most divergent gene segment, and it was found to have an older ancestral relationship to known influenza A and B viruses. The solution of the N10 crystal structure determined that, although it shares general structural features with the other influenza A

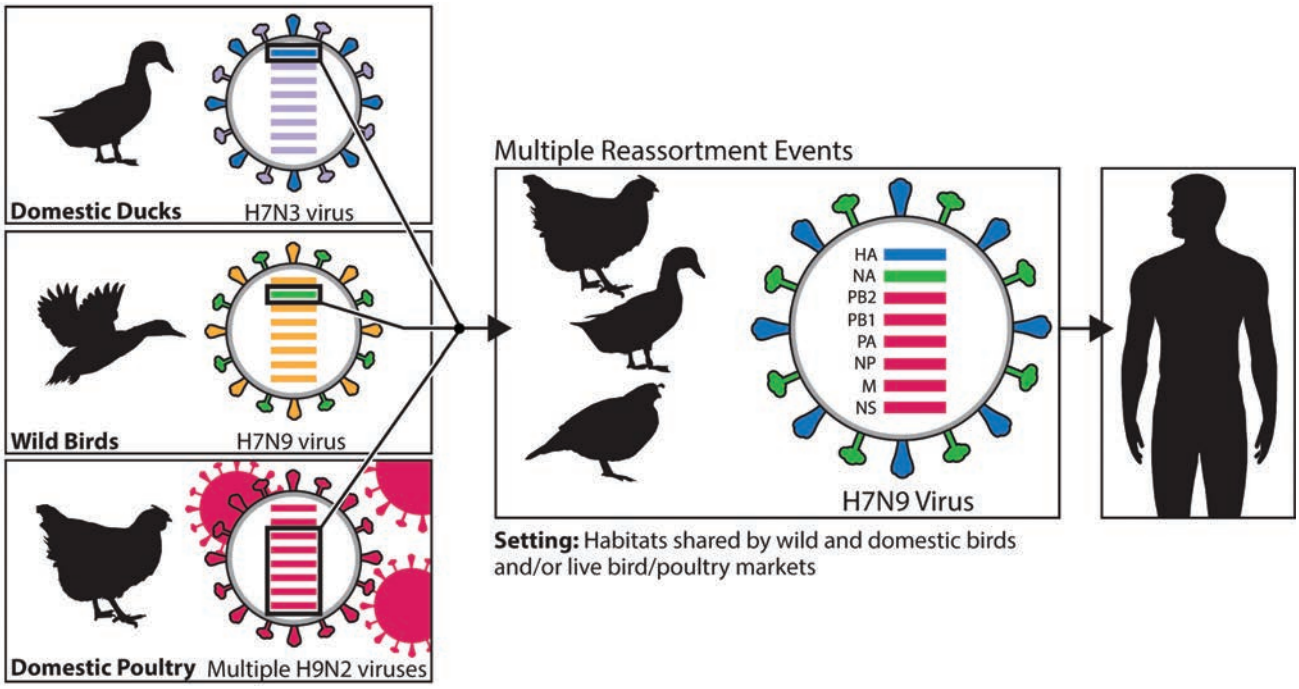


Figure 25-11. Diagram showing the likely genetic evolution of the H7N9 virus that emerged in China in 2013. The eight genes of the H7N9 virus are closely related avian influenza viruses found in domestic ducks, wild birds, and domestic poultry in Asia. The virus likely emerged from "reassortment," a process in which two or more influenza viruses coinfect a single host and exchange genes. This process can result in the creation of a new influenza virus, and it is likely that multiple reassortment events led to the creation of the H7N9 virus. These events may have occurred in habitats shared by wild and domestic birds and/or in live bird/poultry markets, where different species of birds are bought and sold for food. As the above diagram shows, the H7N9 virus likely obtained its HA (hemagglutinin) gene from domestic ducks, its NA (neuraminidase) gene from wild birds, and its six remaining genes from multiple-related H9N2 influenza viruses in domestic poultry.
M: matrix; NP: nucleoprotein; NS: nonstructural; PA: polymerase subunit A; PB1: polymerase subunit B1; PB2: polymerase subunit B2
Diagram: Courtesy of Centers for Disease Control and Prevention.

NAs whose structures have been determined, it does not have the conserved amino acids that are involved in sialic acid binding and cleavage[210,211] and the protein does not display enzymatic neuraminidase activity necessary for its function in mediating spread of the virus from infected cells. The HA structure suggests that the H17N10 virus does not use sialic acid as a receptor.[179] The polymerase complex encoded by the sequences found in the bats did function in human cells, but the sequences of the polymerase genes suggest that they may be incompatible with other influenza A subtypes.

In 2013, Tong et al[145] reported the identification of RNA encoding another distinct influenza virus, designated H18N11, in flat-faced fruit bats in Peru. Again, the sequences were highly divergent from known influenza A viruses, and they indicated a long-standing virus–host relationship. The sequences were most closely related to the H17N10 influenza sequences previously reported by this group.[144] Structural and functional studies of the HA and NA encoded by these sequences suggest that sialic acid is not a receptor for the virus and is not used for virus release from the infected cell. The H18N11 influenza virus was not isolated, but viral sequences were identified in rectal swabs and intestines of the bats. The overall H18 structure was found to be similar to that of the known influenza A trimers, but unlike the known HAs, the H18 structure infers no requirement of low pH for fusion. In addition, the receptor-binding domain of the H18 glycoprotein is dramatically different. Like the N10 NA, the general N11 structure is similar to the known influenza A NAs (ie, tetrameric), but the N11 active site is different, even from that of the N10 protein. The N11 protein does not display glycan binding or enzymatic neuraminidase activity. Seroprevalence studies found that approximately 38% of the Guatemalan bats tested had detectable antibodies to H17,[144] and 50% of bats tested had antibodies to either the recombinant H18 or N11.[145]

Some debate exists as to whether the viruses encoded by the viral sequences identified in the bats in Guatemala and Peru should even be classified as influenza A viruses. They may represent ancient ancestral viruses. The significance of these findings for the potential for the emergence of novel influenza viruses that may infect humans, or for reassortment with other influenza viruses in nature remains to be determined.

Diseases Caused by Emerging Coronaviruses

SARS, which first emerged in Guangdong province of China in November 2002, is a classic example of a newly emerging viral disease. By January 2003, the disease had spread to Guangzhou, the capital of Guangdong province, and caused major outbreaks, primarily affecting healthcare workers. In February 2003, a physician from Guangdong spent a single day in a hotel in Hong Kong, during which time he transmitted the infection to 16 other guests. These individuals quickly spread the disease to Hong Kong, Singapore, Vietnam, and Toronto.[212] Within weeks, SARS had spread to affect thousands of people in 25 countries across five continents and, by the end of the global outbreak (July 2003), more than 8,000 reported cases existed, with 744 fatalities.[213] Within 4 months of the beginning of the outbreak, a novel coronavirus (SARS-CoV) was identified as the infectious agent of the syndrome.[214–216] Cases of SARS have not been reported since 2003.

Middle Eastern Respiratory Syndrome

In June 2012, a 60-year-old man was admitted with a history of fever, cough, expectoration, and shortness of breath to a hospital in Jeddah, Saudi Arabia.[217] Despite treatment in an intensive care unit, the patient died 11 days after admission from respiratory and renal failure. Clinical isolates were initially tested and found negative for influenza, parainfluenza, enterovirus, and adenovirus. A sputum sample that was obtained upon admission was inoculated in Vero and LLC-MK2 cells, resulting in visible cytopathic effect. A viral family-wide PCR performed on nucleic acid extracted from infected cells gave a positive result for coronaviruses. Sequencing of the PCR amplicon resulted in a novel sequence that indicated the newly discovered virus was most closely related to bat coronaviruses. The first report of the novel coronavirus was made in ProMED-mail on September 20, 2012, by Dr Ali Mohamed Zaki of the Dr Soliman Fakeeh Hospital.[218]

Virus samples were sent for full genome deep sequencing to the Erasmus Medical Center in Rotterdam, Netherlands.[219] Full-genome sequencing confirmed that the novel virus was similar to BtCoV-HKU4 and BtCoV-HKU5, members of the C lineage of the betacoronavirus, but it was sufficiently different enough to warrant classification as a new species that was named HCoV-EMC/2012.

Coronaviruses have relatively large, single-stranded, positive-sense RNA genomes. The HCoV-EMC/2012 genome is approximately 30 kb in length and encodes both structural and nonstructural proteins. Before 2003, only two coronaviruses (HCoV-229E and HCoV-OC43) were known to infect humans, and those caused only mild respiratory disease.[220,221] As noted previously, SARS-CoV previously caused an epidemic in 32 countries, infecting more than 8,000 people. Since 2003, two additional human coronaviruses have been identified, HCoV-NL63[177,222] and HCoV-HKU1,[223] both of which can cause pneumonia.

On September 23, 2012, the United Kingdom Health Protection Agency reported on the case of a 49-year-old man who had become sick while in Saudi Arabia in August 2012. That illness resolved, but he subsequently presented to a physician in Qatar with a cough, myalgia, and arthralgia on September 3, 2012. Five days later he was admitted to the hospital with fever and hypoxia. His condition worsened and he was transferred to London by air ambulance. His condition deteriorated once in London, and he was placed on extracorporeal membrane oxygenation on September 20.[144] Initially, the patient was screened for common viral and bacteriological causes of respiratory illness with no positive results. After the September 20 ProMED report of a novel coronavirus identified in the Middle East, patient samples were screened by using a pan-coronavirus reverse transcriptase polymerase chain reaction (RT-PCR) assay. Sequencing of the PCR product showed it was nearly identical to the EMC/2012 virus.

Following the initial description of these two cases which came to be called Middle East Respiratory Syndrome (MERS), a retrospective investigation of an outbreak of acute respiratory disease was performed at the Zarqa hospital in Jordan.[224] In April 2012, the Zarqa hospital had 13 patients who presented with high fever and acute lower respiratory symptoms. Laboratory tests performed at the time of the outbreak were inconclusive. The cluster consisted of two phases. In the first phase, four patients had onset of symptoms between March 21 and April 2. The patient with the earliest onset (a 25-year-old student) and a 40-year-old nurse who worked at the hospital died within 2 to 4 weeks of symptom onset. MERS-CoV infection was confirmed in both of these cases by specific RT-PCR. A second wave of disease followed with onset of symptoms between April 11 and April 26. This second

wave consisted of seven healthcare workers from the hospital and two family members of patients from the first wave of disease. Three of the healthcare workers and the two family members had close contact with individuals in the first wave, raising the possibility of limited person-to-person transmission of the virus.

A published report in June 2013 confirmed person-to-person transmission in a cluster of 23 confirmed and 11 probable MERS-CoV infections in hospitals in the Al-Hasa governorate of Saudi Arabia.[200] The first patient was admitted to the hospital on April 5, 2013, with dizziness and diaphoresis. He was not tested for MERS-CoV, but infection was subsequently confirmed in his son. The first patient is thought to have transmitted the virus to a patient in an adjacent room (in addition to his son), who then transmitted the virus to an additional seven patients (six in the dialysis unit and one in the intensive care unit). Further transmission of the virus was documented to an additional 10 patients, two healthcare workers, and three family members.

In May 2015, Republic of Korea health officials reported a case of MERS-CoV infection in a 68-year-old man who had been traveling in the Middle East for several weeks. He was asymptomatic while traveling,

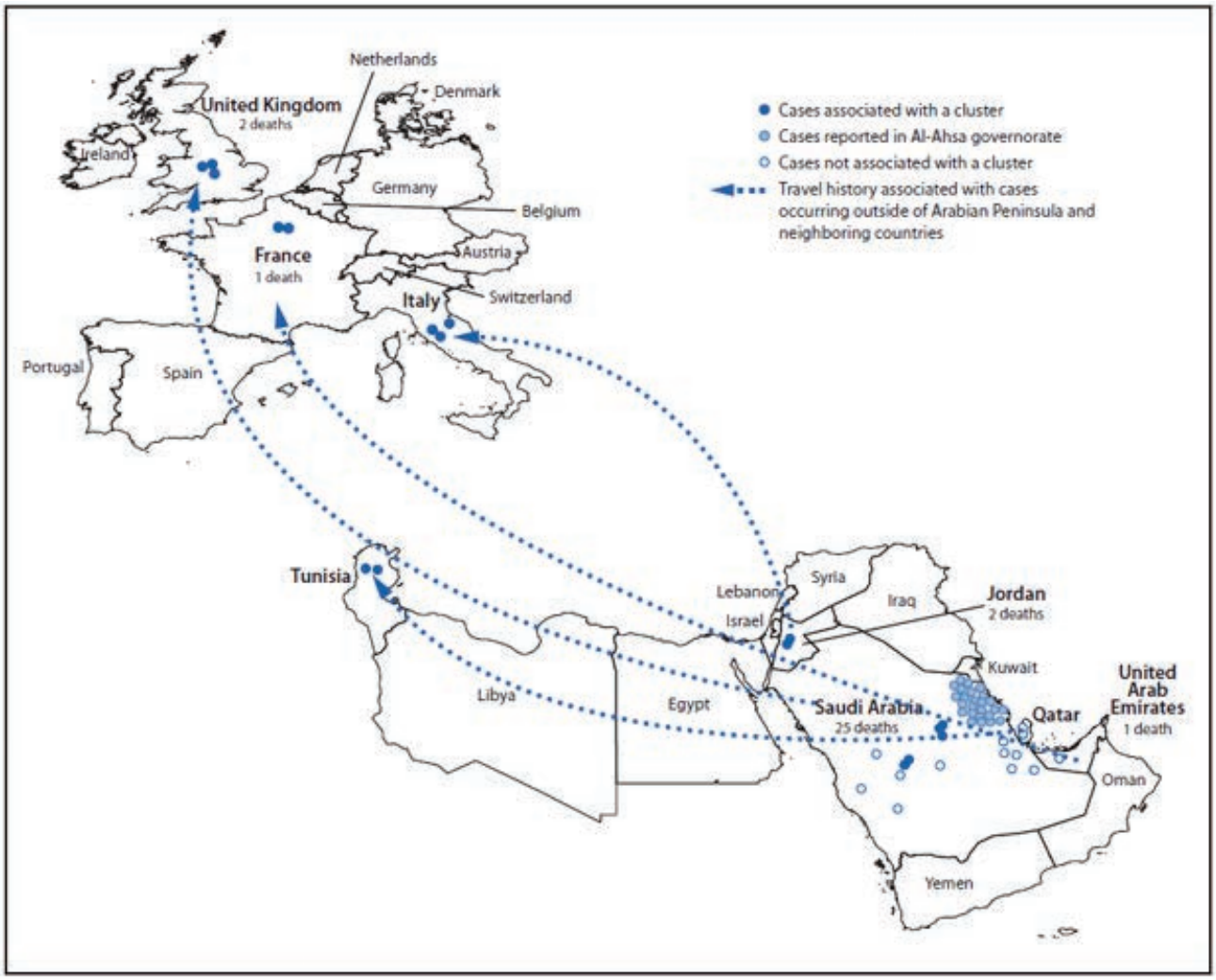


Figure 25-12. History of travel from in or near the Arabian Peninsula within 14 days of illness onset for confirmed cases (N = 130) of Middle East respiratory syndrome coronavirus infection reported to the World Health Organization from 2012 to 2013. All cases have been directly or indirectly linked through travel to or residence in Saudi Arabia, Qatar, Jordan, and the United Arab Emirates. Figure does not include recent cases in South Korea.
Data source: Centers for Disease Control and Prevention. Updated information on the epidemiology of Middle East respiratory syndrome coronavirus (MERS-CoV) infection and guidance for the public, clinicians, and public health authorities, 2012–2013. *MMWR Morb Mortal Wkly Rep*. 2013;62:793–796.

but developed symptoms within a week of returning to Korea. He was seen at four separate hospitals and was admitted to the fourth one in Seoul on May 18, where it was confirmed that he was infected with MERS-CoV.[225] During his medical visits, before being diagnosed, he infected around 30 other individuals who were present in the hospital at the same time. A secondary case from the second hospital went on to infect more than 80 additional people. In total, 186 cases of MERS (185 in the Republic of Korea and 1 in China[226]) were confirmed during the Korean outbreak, more than half of which had been infected by one of the two "superspreaders."

As of September 2, 2015, 1,493 confirmed cases of MERS-CoV infection resulted in 527 deaths (35% case fatality rate).[226] The majority of these cases were from Saudi Arabia, but Korea, France, Italy, Jordan, Qatar, Tunisia, the United Kingdom, and the United Arab Emirates also have reported cases. During the recent outbreak in South Korea, secondary and tertiary transmission occurred from the index case who traveled to the Middle East. All other cases outside of the Middle East had recorded recent travel to the Middle East (Figure 25-12).[227]

A meta-analysis of 47 laboratory-confirmed cases of MERS-CoV infection from Saudi Arabia found that most patients presented with fever, cough, shortness of breath, and myalgia.[200] Almost all of the identified patients had underlying comorbidities, including diabetes (68%), chronic kidney disease (49%), hypertension (34%), chronic heart disease (28%), and chronic lung disease (26%). Twenty-eight (60%) of the patients died, and the fatality rate increased with increasing age.

The receptor for MERS-CoV was identified within 6 months of the original characterization of the virus. Dipeptidyl peptidase 4 (DPP4) from extracts of cells susceptible to virus infection co-purified with the receptor-binding domain of the EMC/2012 spike protein.[228] Transient expression of DPP4 in nonsusceptible cells also rendered them susceptible to infection and preincubation of cells with anti-DPP4 polyclonal antibodies made them resistant to MERS-CoV infection. The rapid identification of the receptor opens several avenues for generating antiviral therapeutics. Manipulation of DPP4 levels or the development of small molecules or monoclonal antibodies that can block the interaction of MERS-CoV with DPP4 could potentially alter the course of disease in infected individuals.

Other therapeutic interventions may prove useful for treatment of MERS-CoV infection. The virus has been shown to be sensitive to type I interferon in vitro, with its replication limited by two to four orders of magnitude when cells are pretreated with interferon.[229,230] In addition, in a rhesus macaque model of MERS-CoV infection,[231] treatment with interferon α2b and ribavirin was shown to ameliorate some of the disease symptoms.[231] Additionally, a monoclonal antibody isolated from an infected patient has shown prophylactic and postexposure protection against MERS-CoV infection in a mouse model of infection.[232] Vaccination of mice, camels, and rhesus macaques with a DNA vaccine expressing the MERS-CoV spike protein was shown to elicit neutralizing antibody responses in all three species, and it could also protect macaques from challenge with MERS-CoV.[233]

Parallels can be drawn between the current MERS-CoV epidemic and the SARS epidemic of 2002 to 2003. Although the reservoir for MERS-CoV has not been identified, both viruses are thought to circulate in bats. SARS-like coronaviruses were identified in three species of bats from the genus *Rhinolophus*.[186] The virus may have spilled over into palm civets (*Paguma larvata*) (Figure 25-13), which served as an amplifying host. The virus could then be transmitted to humans when they came into contact with infected civets in wild animal markets. The genome sequence of MERS-CoV indicates that it is closely related to two bat coronaviruses, BtCoV-HKU4 and BtCoV-HKU5. A bat survey conducted in Saudi Arabia identified a fecal sample from a *Taphozous perforatus* bat that yielded a PCR product with 100% identity to the sequence of MERS-



Figure 25-13. The masked palm civet was originally implicated as the possible animal source for the SARS coronavirus after SARS-like coronaviruses were isolated from animals found in a live animal market in Guangdong, China. These animals are trapped and butchered for food in southern China. This photograph was taken at a wet market in Guangzhou in May 2003.
SARS: severe acute respiratory syndrome
Photograph: Courtesy of Dr Meirion Evans, Cardiff University, United Kingdom.

CoV EMC/2012.[234] In another survey, a fecal sample from a South African *Neoromicia zuluensis* bat yielded a PCR product whose nucleotide sequence indicated that it was closely related to the MERS-CoV.[235]

Although MERS-CoV-like viruses have been identified in bats, nothing indicates that MERS-CoV is jumping directly from bats into humans. One possibility is that MERS-CoV may move from bats through an intermediate host that has a closer association with humans, as was the case of the SARS-CoV. Serum surveys of livestock in Egypt, Oman, and Spain identified high levels of MERS-CoV reactive antibodies in dromedary camels.[236,237] Subsequently, MERS-CoV RNA was detected in three camels that had close association with two human cases.[218] In November and December 2013, a large, nationwide serosurvey of livestock in the Kingdom of Saudi Arabia found that 74% of the sampled dromedary camels had antibodies reactive to MERS-CoV.[238] Testing of archived serum samples found MERS-CoV reactivity dating back to 1992, indicating that the virus has been circulating in the Kingdom of Saudi Arabia since at least that time. In addition to the serological data, two groups isolated replication-competent MERS-CoV from dromedary camels in late 2013 through early 2014.[239,240]

Public health measures were an important aspect of halting the spread of the SARS epidemic, and public health officials worldwide have been proactive with measures intended to reduce the possibility that MERS could become another pandemic. One of the main concerns has centered on the Hajj, the annual event in which millions of Muslim pilgrims from around the world travel to Mecca in Saudi Arabia. The Saudi Arabian Ministry of Health recommended that persons older than 65, pregnant women, children younger than 12, or those with chronic diseases should postpone performing the Hajj in 2013. Many may have heeded those warnings, as participation in the 2013 Hajj was estimated at just less than 2 million pilgrims, down from 3.2 million pilgrims in 2012.

As of this writing, cases of MERS are still occurring on the Arabian Peninsula. Unfortunately, unlike SARS-CoV, MERS-CoV shows no sign of abating and continued efforts to understand this virus will be important to control this emerging disease.

Diseases Caused by Emerging Paramyxoviruses

Hendra Virus

In 1994, a new member of the paramyxoviruses emerged for the first time in Brisbane, Australia, killing 14 race horses and a horse trainer.[241] Another worker at the stable survived with an influenza-like illness. One year later, a farmer from Mackay (800 km north of Brisbane) died as a result of encephalitis caused by this novel virus.[242] Two of his horses were subsequently shown to have died from the same virus 13 months earlier. Genetic analysis of the virus showed it was distantly related to the morbilliviruses, which contain other members such as rinderpest, measles, and canine distemper viruses, and so the virus was initially named equine morbillivirus,[241] but was later renamed Hendra virus after the Brisbane suburb where the outbreak occurred. Serologic evidence[243] and later evidence of infection was found in several species of Australian flying foxes (ie, fruit bats of the genus *Pteropus*) (Figure 25-14), supporting epidemiological evidence that fruit bats are the natural reservoir for Hendra virus. Field, experimental, and molecular investigations indicate that Hendra virus is an endemic fruit bat virus that has probably co-evolved with its pteropid hosts.[53,244,245]

Although additional occurrences of Hendra virus have been relatively rare and sporadic, as of June 2014, 50 outbreaks of Hendra virus occurred in Australia, all involving infection of horses. Four of these outbreaks have spread to humans as a result of direct contact with infected horses. The case fatality rate in humans is 60% and in horses 75%.[246]

Nipah Virus

Nearly 5 years after the discovery of the Hendra virus, a massive outbreak of porcine respiratory disease occurred in Malaysia and subsequently caused the deaths of 105 pig farm or abattoir workers, the eventual culling of more than 1 million pigs, and the discovery of a new virus closely related to Hendra called Nipah virus.[247] The predominant clinical syndrome in humans was encephalitic (not respiratory as was seen in the infected pigs) with clinical signs including fever, headache, myalgia, drowsiness, and disorientation sometimes leading to coma within 48 hours.[248,249] The majority of human cases had a history of direct contact with infected pigs, most of whom were pig farmers. Preliminary research on the new virus revealed that it had ultrastructural, antigenic, serologic, and molecular characteristics similar to Hendra virus.[247] Follow-up molecular studies showed the genome of Nipah virus to be highly homologous to that of Hendra virus, with specific genes sharing from 70% to 88% nucleotide homologies and amino acid homologies ranging from 67% to 92%.[247] Given the degree of similarity and other unique features of these viruses, they both were placed in a new genus, *Henipavirus*, within the family *Paramyxoviridae*.[250] With the knowledge of the similarities between Nipah and Hendra viruses, it was natural that attention focused on Malaysian bats as the source of the

infection in pigs.[53] Initial surveillance efforts identified the presence of neutralizing antibodies to Nipah virus in the sera of 21 bats from five species (four species of fruit bat, including two flying fox species, and one insectivorous bat species).[251] Although no virus was isolated or viral RNA amplified from these seropositive bats, later attempts proved successful and virus was isolated from pooled urine samples collected from a colony of seropositive flying foxes from Tioman Island off the coast of Malaysia.[252]

The virus reemerged in Bangladesh in 2001, each resulting in a cluster of febrile neurologic illnesses with nine reported deaths.[253] Since 2001, outbreaks of Nipah have occurred nearly every year in Bangladesh. More than 70% of those infected have died and one-third of the survivors have permanent neurological deficits.[254] Outbreak investigations in Bangladesh have identified consumption of raw date palm sap as the primary route of transmission of Nipah virus from *Pteropus* bats to people. Date palm sap is harvested in the winter in Bangladesh by shaving the bark from the sugar date palm tree and collecting the sap into open clay pots. *Pteropus* bats (see Figure 25-14) that shed Nipah virus in their saliva frequently visit the trees during sap collection and lick the sap as it is running into the pot, thereby contaminating the sap.[255] However, similar to other viruses, such as Ebola virus, transmission can also occur by direct contact with infected individuals, particularly during patient care (ie, nosocomial transmission) or exposure to infected patients' bodily secretions during traditional burial practices.[256] Thus, patients from regions where Nipah virus is known to occur who present with meningoencephalitis should be placed in an isolation room or ward and healthcare workers caring for these patients should wear gloves and masks.

Emerging Mosquitoborne Viruses: Dengue, West Nile, and Chikungunya

Mosquitoborne viruses are members of the more general category of arthropodborne viruses or arboviruses. Human infection with arboviruses can be asymptomatic or can cause diseases ranging from a mild febrile illness to encephalitis or even severe hemorrhagic fever in some cases. Others cause rash and epidemic arthralgia. Most arboviruses require a reservoir host such as a bird or small mammal while using a vector—usually a mosquito or tick—for transmission to another host.[257] From this complex life cycle, many arboviruses are restricted to specific geographical regions. For example, Ross River and Murray Valley encephalitis viruses are restricted to Australia and surrounding islands, whereas o'nyong-nyong virus

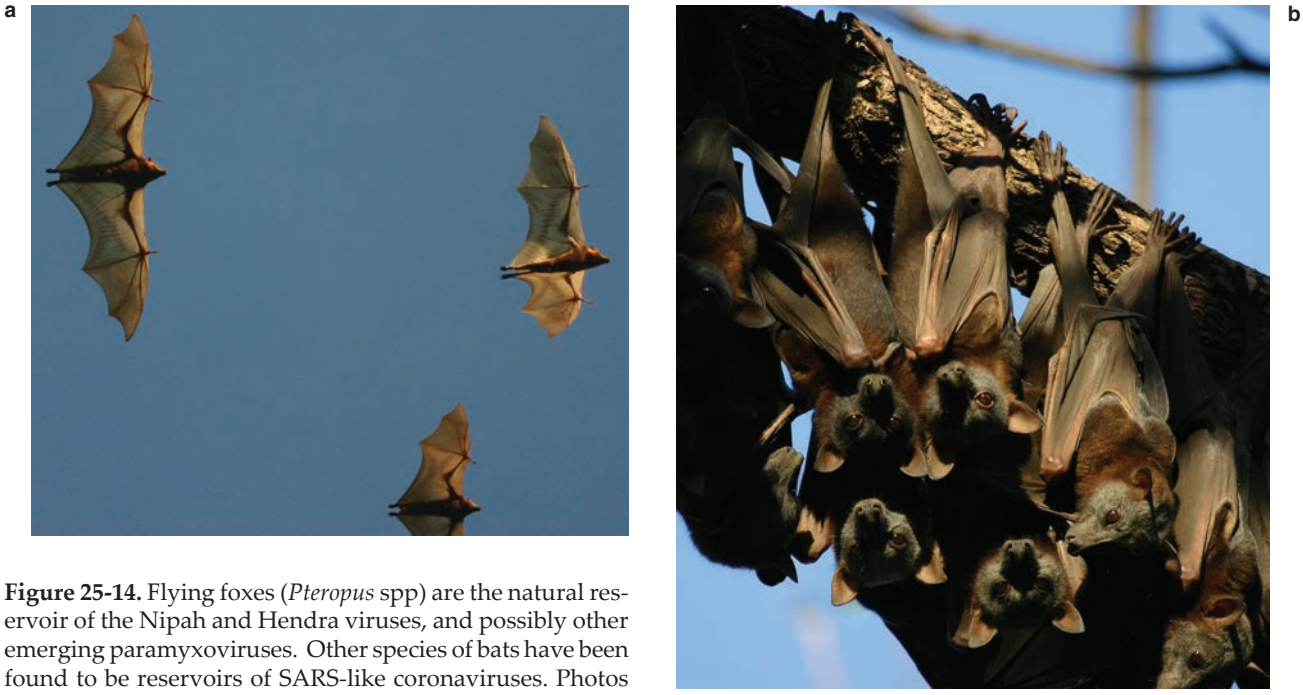


Figure 25-14. Flying foxes (*Pteropus* spp) are the natural reservoir of the Nipah and Hendra viruses, and possibly other emerging paramyxoviruses. Other species of bats have been found to be reservoirs of SARS-like coronaviruses. Photos show the little red flying fox (*Pteropus scapulatus*) in flight (**a**) and roosting (**b**).
Photographs: Courtesy of Raina Plowright, Department of Veterinary Medicine and Epidemiology, University of California, Davis, California.

occurs only in Africa. However, because of various ecological or environmental changes (whether natural or manmade) that lead to changes in the mosquito vector distribution or genetic changes in the viruses themselves, some arboviruses may not always remain restricted to their previously known geographical regions.

Dengue Virus

Dengue fever, which is caused by one of four viral subtypes (designated DENV-1 to DENV-4), is one of the most common mosquitoborne viral infections of humans, with up to 100 million cases reported annually and some 2.5 billion people living at risk of infection in tropical and subtropical regions of Africa, Asia, and the Americas.[258] Infection with dengue virus (DENV) can present in several clinical manifestations. Classical dengue fever is an acute febrile illness that often occurs in children and is characterized by fever, severe headache and muscle aches, nausea, vomiting, and rash. This acute illness, which usually lasts for 8 to 10 days, is rarely fatal. A more severe form of dengue infection is dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). DHF usually begins during the first week of the acute illness and can lead to hemorrhagic manifestations, including petechiae, ecchymoses, epistaxis, bleeding gums, and gastrointestinal tract bleeding.[259] DSS occurs if the patient goes on to develop hypotension and shock due to plasma leakage and circulatory failure, which happens in about one-third of severe dengue cases (especially children) and is often associated with higher mortality. Convalescence for patients with DHF is usually short and uneventful, and if shock is overcome, patients usually recover within 2 to 3 days.[259]

The pathogenesis of DHF/DSS is complicated and not well understood. Two theories are frequently cited to explain the pathogenetic changes that occur in DHF/DSS. The most commonly accepted theory is known as immune enhancement.[260,261] This idea suggests that patients experiencing a second infection with a heterologous DENV serotype have a significantly higher risk of developing DHF and DSS. Preexisting heterologous dengue antibody recognizes the infecting virus and forms an antigen-antibody complex, which is then bound to and internalized by immunoglobulin Fc receptors on macrophages. Thus, it is hypothesized that prior infection, through a process known as antibody-dependent enhancement, enhances the infection and replication of DENV in mononuclear cells.[259] The other theory assumes that DENV changes genetically as a result of selective pressures as it replicates in humans and/or mosquitoes and that the phenotypic expression of these genetic changes may include increased virus replication and virulence. All the data suggest that a combination of one's viral, immunopathogenic, age, and genetic background plays a role in disease severity.[258]

Although first identified in southeast Asia in the 1940s and 1950s, evidence suggest that DENVs were derived from a primitive progenitor introduced to Asia from Africa about 1,000 years ago.[262] Studies of DENV ecology in sylvatic habitats of west Africa and Malaysia have identified transmission cycles involving nonhuman primates as reservoir hosts and arboreal, tree-hole dwelling *Aedes* species mosquitoes as vectors.[263,264] Efficient interhuman DENV transmission probably requires a human population of 10,000 to 1 million people, a feature of urban civilization that did not exist until about 4,000 years ago, suggesting the sylvatic cycle is probably ancestral.[265] Further support for this idea comes from studies suggesting that a zoonotic transfer of DENV from sylvatic to sustained human transmission occurred between 125 and 320 years ago.[262] In the past 300 years, these viruses have become established in the urban centers of the tropics. The principal urban vector, *Aedes aegypti*, is highly domesticated and is adapted to humans, preferring to feed on them and lay their eggs in artificial containers in and around houses. *Ae. albopictus* (the Asian tiger mosquito) (Figure 25-15) is a secondary vector of DENVs.

In the past 25 years, a marked global emergence of epidemic dengue has occurred, with more frequent and larger epidemics associated with more severe disease.[259,266,267] The reasons are not fully understood, but are thought to stem from major demographic and



Figure 25-15. A female *Aedes albopictus* mosquito feeding on a human host. This mosquito, along with *Aedes aegypti*, are competent vectors of dengue virus.
Photograph: Courtesy of James Gathany, Centers for Disease Control and Prevention Public Health Image Library. Image 4490.

societal changes that have occurred over the past 50 years. In particular, unprecedented global population growth and associated unplanned and uncontrolled urbanization occurred, especially in the tropical developing countries.[259] Other potential factors associated with the global emergence of dengue include the lack of effective mosquito control in many tropical areas where dengue is endemic, increased international air travel, and a general decay in public health infrastructure in most countries over the past 30 years.[259] Dengue does occur—albeit rarely—in the United States, primarily in southern Texas and Florida. Because the mosquito vectors that transmit DENVs are distributed throughout much of the southeastern United States, there is likely a greater potential for emergence of dengue. This situation may be unfolding in southern Florida.[267a] Florida has a history of epidemic DENV transmission, but more recent cases were most likely imported by tourism or triggered by infected individuals traveling into the area. However, in the late summer of 2009, DENV-1 infection was confirmed in a person who acquired the virus while traveling to Key West in Monroe County, Florida. DENV-1 infections were subsequently confirmed in two Monroe County residents without history of recent travel.[268] In 2010, additional dengue cases from Monroe County were reported, and DENV-1 was isolated from a mosquito pool[269] and a blood donor from Key West.[270] Phylogenetic analyses of these viral isolates indicated that endemic DENV-1 was transmitted in Key West over at least a 2-year period.[268] In 2013, Martin County in east-central Florida reported 29 cases of locally transmitted DENV-1, and occasional locally transmitted cases have continued to occur since then.[267a]

West Nile Virus

West Nile virus (WNV) was first isolated in 1937 from the blood of a febrile patient in the West Nile district of northern Uganda. It is now one of the most widely distributed of all mosquitoborne arboviruses, and it is found in areas throughout Africa, Europe, Asia, and the Americas. Yet, until 1999, it was completely exotic to the Western Hemisphere. In the late summer of 1999, WNV emerged in the New York City area as the cause of an outbreak of meningoencephalitis resulting in 7 deaths among 62 confirmed cases.[75] A concurrent outbreak occurred among the horse population on Long Island, resulting in 25 equine cases, including 9 fatalities.[271] The principal mosquito vectors were *Culex pipiens* or other related *Culex* species; however, the virus has been isolated from other mosquito species and even from ticks in some cases.[266,272] The virus has been shown to be capable of infecting more than 50 species of mosquitoes and ticks.[272,273] Since the introduction of WNV into New York in 1999, the virus

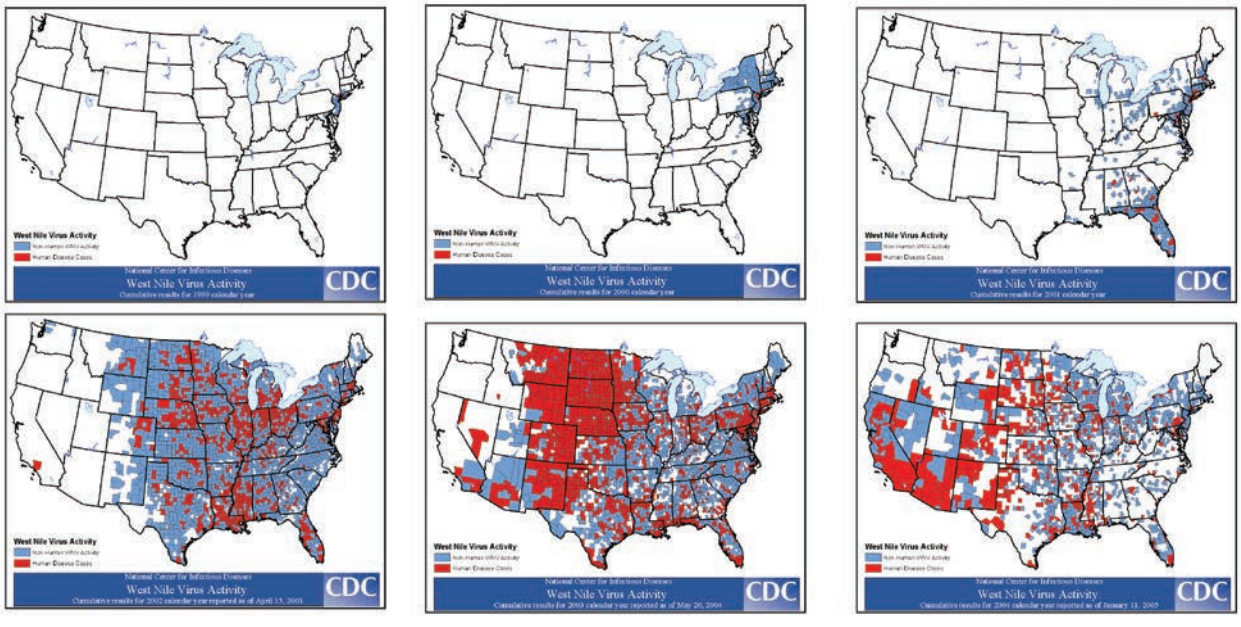


Figure 25-16. Yearly spread of West Nile virus activity across the United States, 1999 to 2004. Data represent counties reporting West Nile virus activity in humans (*red*) and nonhuman (eg, birds, mosquitoes, equines, and other mammals) (*blue*) in the United States.
Data source: National Center for Infectious Diseases, Centers for Disease Control and Prevention.

has spread across the United States (Figure 25-16), north into Canada, and south into Mexico, Central America, and the Caribbean Islands.

Recent years have seen a high incidence of human infection with WNV through blood transfusion, mother-to-fetus transmission, and transmission in breast milk, and also by organ transplantation, causing even greater public health concerns.[96,212,274,275] After several years of low WNV activity in the United States, a multistate outbreak was seen in 2012, with more than 5,600 cases and 286 deaths recorded.[276]

Chikungunya

The first recorded outbreak of chikungunya (CHIK) occurred in the Newala District of Tanzania (formerly Tanganyika) in 1952 to 1953.[277,278] The outbreak was initially thought to have been caused by DENV because it shared many clinical features with dengue infection and was thought to be transmitted by *Ae aegypti* mosquitoes. The infection manifested with a sudden onset of incapacitating joint pain and high fever, leading locals to call it chikungunya, meaning "that which bends up" in the local Makonde language. The disease also often led to development of a maculopapular rash, anorexia, and constipation. Most symptoms usually resolved within 7 to 10 days, but the arthralgia could last for months following the infection. In some patients, the joint pain was so severe months after infection that they were unable to change position without help.

A viral agent was recovered from the serum of acutely ill patients by intracerebral inoculation into mice.[279] Hyperimmune serum raised against the virus could cross neutralize Semliki Forest virus (an alphavirus) but not DENV, indicating that the virus was more closely related to the alphaviruses than flaviviruses.

The virus isolated from the outbreak in the Newala District, chikungunya virus (CHIKV), is an Old World

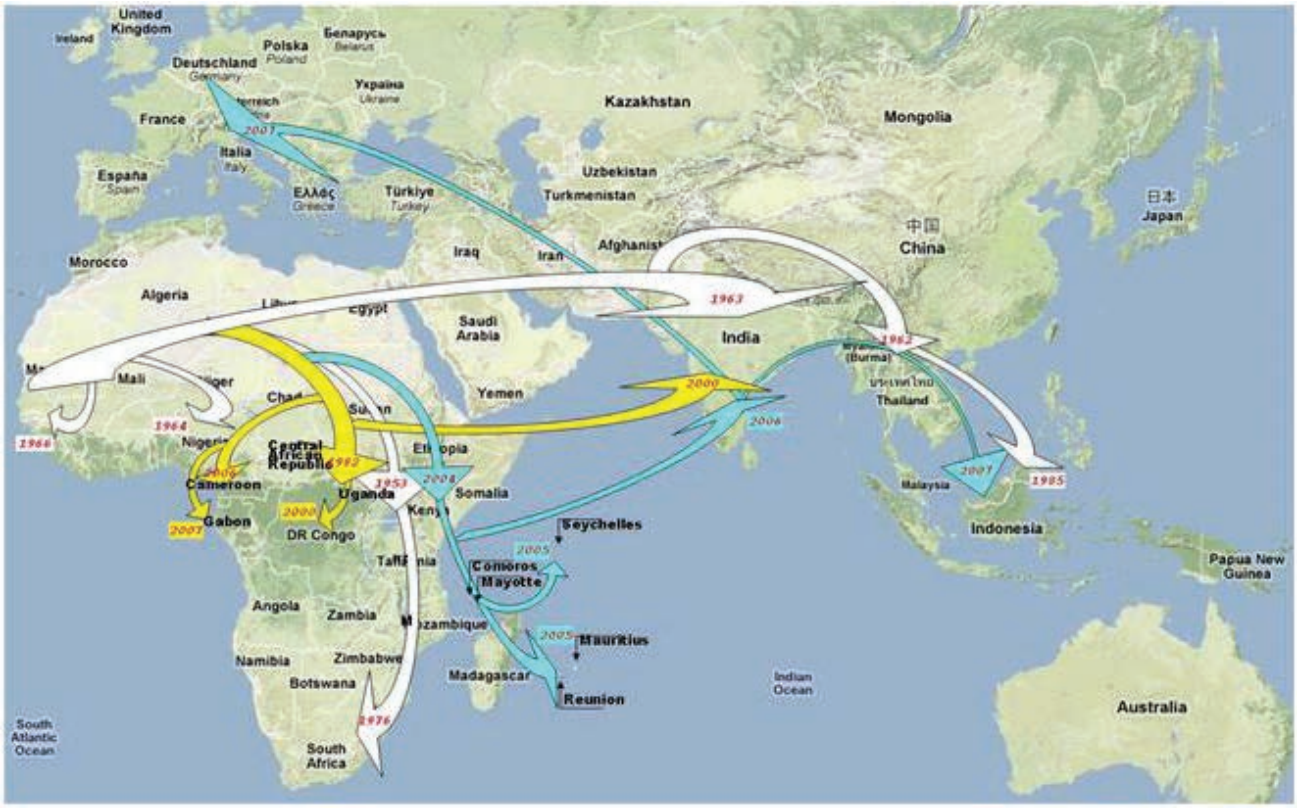


Figure 25-17. Predicted dispersal pattern of chikungunya virus from Africa to the Indian Ocean and Europe during the past 20 to 50 years.
DR Congo: Democratic Republic of the Congo
Photograph: Courtesy of Creative Commons, licensed under CC BY 2.0. https://virologyj.biomedcentral.com/articles/10.1186/1743-422X-5-33.
Data source: de Lamballerie X, Leroy E, Charrel RN, Ttsetsarkin K, Higgs S, Gould EA. Chikungunya virus adapts to tiger mosquito via evolutionary convergence: a sign of things to come? *Virol J*. 2008;5:33.

alphavirus in the Semliki Forest antigenic complex that is found mainly in Africa and Southeast Asia. African transmission follows a sylvatic cycle between nonhuman primates, small mammals, and *Aedes* species mosquitoes, with occasional spillover into human populations when vector populations are high.[263] Asian transmission follows an urban cycle, with the virus transmitted between humans via the urban dwelling *Ae aegypti* and *Ae albopictus* mosquito vectors.

Between 1960 and 1980 numerous documented outbreaks of CHIK occurred throughout Africa and Asia, followed by relative quiet between 1980 and 2000.[280] In 2000, the virus reemerged when an estimated 50,000 people were infected in Kinshasa, Democratic Republic of the Congo, the first reappearance of the virus there in 39 years.[281,282] From May until July 2004, an outbreak occurred on Lamu Island off the coast of Kenya. There were 1,300 reported cases of CHIKV infection of a total population of 18,000 on the island. A seroprevalence study conducted after the epidemic found that 75% of the population had detectable IgG and/or IgM antibodies to the virus, indicating that approximately 13,500 people had been infected.[283] The virus spread to Mombasa, Kenya, and then to the Comoros Islands, where an estimated 215,000 people contracted the disease on Grand Comore Island between February and May of 2005.[284]

Additional outbreaks occurred on the Indian Ocean islands of Mauritius, the Seychelles, Madagascar, and Mayotte, culminating in a large outbreak on Reunion Island between March 1, 2005 and April 30, 2006. During the Reunion Island outbreak, an estimated 255,000 people were infected.[285] The outbreak on Reunion Island was unusual because the main mosquito vector, *Ae aegypti*, was not abundant on the island. It appeared that the main vector responsible for transmission during the Reunion outbreak was *Ae albopictus*. Genetic characterization of the virus from Reunion Island identified a key single amino acid change (A226V, ie, the alanine at position 226 was changed to valine) in the envelope glycoprotein that enabled the virus to infect *Ae albopictus* more efficiently.[286,287]

A large outbreak of CHIKV occurred in India in 2006, marking a return of the virus that had been absent for 33 years (Figure 25-17; note: the *yellow arrow* indicating the presence of CHIKV in India in 2000 is derived from a virus isolated from mosquitoes in Yawat, Maharashtra, not a human case). It is estimated that 1.4 million people were infected. Genetic analysis of the virus showed that it was related to the East African and Indian Ocean strains from the previous couple of years, but it lacked the A226V mutation.[288] During a second wave of infection in 2007 in Kerala, India, the virus had obtained the A226V mutation (likely independently from the Reunion strains of the virus), indicating that it had adapted to the high population densities of *Ae albopictus* in the area at the time.[288,289]

The rapid adaptation of CHIKV to *Ae albopictus* mosquitoes may represent a threat to Europe and North America. *Ae aegypti* mosquitoes have only been detected in a small swath of the southern United States. *Ae albopictus*, however, has been detected as far north as Pennsylvania, New Jersey, and southern New York in the United States, and as far north as Germany and the Netherlands in Europe. An outbreak in Ravenna, Italy during the summer of 2007 may foreshadow potential future outbreaks in the United States and Europe. A visitor from the active outbreak area of Kerala, India became ill with CHIKV 2 days after arriving in Ravenna on June 21. Virus was transmitted locally by *Ae albopictus* mosquitoes, resulting in 205 autochthonous cases identified between July 4 and September 27, peaking during the third week of August.[290] This was the first observation of sustained CHIKV transmission in a temperate climate.

In December 2013, the WHO reported confirmed cases of CHIKV infection on the Caribbean Island of Saint Martin including two confirmed cases, four probable cases, and another 20 suspected cases.[291] None of the cases reported recent travel outside of Saint Martin, indicating that these were the first reported cases of local transmission of CHIKV in the Western Hemisphere.

After the initial detection in Saint Martin, the virus spread rapidly throughout the Caribbean, and South and Central America. The cumulative case number for 2014 throughout the Americas reached nearly 25,000 confirmed and more than 1.1 million suspected cases, with the highest incidence rate of 56% occurring on the island of Martinique.[292]

In the summer of 2014, the Florida Department of Health reported detection of the first autochthonous transmission of CHIKV in the United States.[293] Eleven cases of transmission were detected in Miami-Dade, Palm Beach, Saint Lucie, and Broward counties.

No treatment is available for CHIKV infection. The sole remedy available consists of treating to alleviate the symptoms. An attenuated CHIKV vaccine developed by the US Army Medical Research Institute for Infectious Disease and the University of Maryland progressed through phase II clinical trials in 2000 before it was discontinued because of a change in funding priorities.[294] The recent explosion in the size of CHIK outbreaks and the demonstration that the virus can cause outbreaks in Europe and potentially the United States may call for a reinvestment in the development of CHIKV vaccines.

Emerging Tickborne Phleboviruses

Until recently, most bunyaviruses (family *Bunyaviridae*) within the genus *Phlebovirus* that were of concern to human health were transmitted by either mosquitoes or sandflies, including viruses such as Rift Valley fever virus or sandfly fever virus, respectively. Recently, new tickborne diseases caused by novel phleboviruses have emerged in China (and later seen in Japan and South Korea) and in the midwestern United States.

Severe Fever With Thrombocytopenia Syndrome Virus

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging tickborne disease first described from rural areas of central China.[295,296] The major clinical symptoms include fever, thrombocytopenia (low platelet count), gastrointestinal symptoms, and leukocytopenia (low white blood cell count). Initial cases (79 cases with 10 deaths, case fatality rate of 12.7%) were found in 2007 from the Henan Province.[295] Interestingly, because of the similarity in clinical symptoms, investigators first suspected human granulocytic anaplasmosis, another tickborne disease caused by the bacterium *Anaplasma phagocytophilum*.[295,296] However, when no bacterial DNA or antibodies against this bacterium could be detected in the blood samples from the majority of the patients, a viral etiology was suspected. In 2009, a novel virus was isolated from the blood of a patient from Xinyang City in Henan Province.[296] Independently, another group identified the virus from the same region of China using high-throughput sequencing of acute-phase sera from 10 patients who had fever, thrombocytopenia, leukopenia, and a history of tick bite.[295] The group also isolated a virus that reacted with patients' sera in immunoflorescence assays and had characteristic virion morphology consistent with that of a bunyavirus (Figure 25-18).[295] These investigators named the disease thrombocytopenia and leukopenia syndrome and the new virus, Henan fever virus, after the location of the index patient.[295] However, SFTS and SFTS virus (SFTSV) are generally accepted.

As the name implies, prominent manifestations of the disease include thrombocytopenia and leukopenia. Other major symptoms include sudden onset of fever and gastrointestinal symptoms (vomiting, diarrhea, and upper abdominal pain).[295,296] Multiorgan failure developed rapidly in most patients as shown by elevated levels of serum alanine aminotransferase, aspartate aminotransferase, creatine kinase, and lactate dehydro-

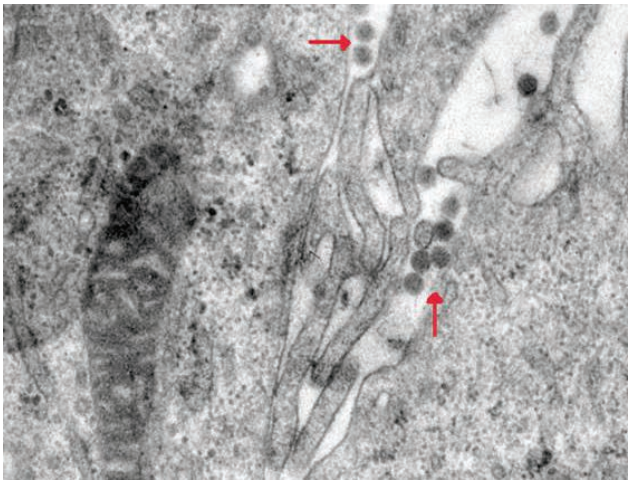


Figure 25-18. Thin-section electron microscopy of the novel bunyavirus (*red arrows*) associated with fever, thrombocytopenia, and leukopenia syndrome (now called severe fever with thrombocytopenia syndrome) in China. Original magnification ×50,000.
Photograph: Courtesy of Creative Commons, licensed under CC BY 2.0. http://journals.plos.org/plospathogens/article?id=10.1371/journal.ppat.1002369.
Data source: Xu B, Liu L, Huang X, et al. Metagenomic analysis of fever, thrombocytopenia and leukopenia syndrome (FTLS) in Henan Province, China: discovery of a new bunyavirus. *PLoS Pathog*. 2011;7:e1002369.

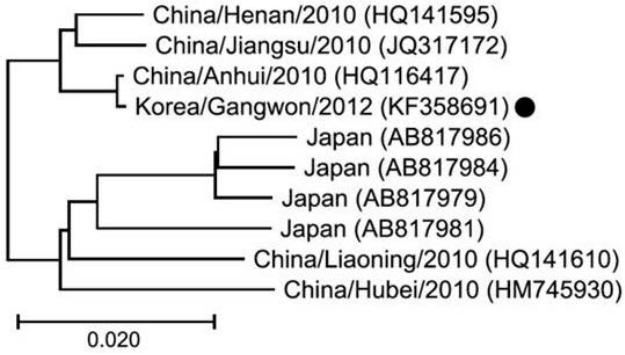


Figure 25-19. Phylogenetic tree for the RNA-dependent RNA polymerase (RdRP) gene sequences of the large segment of an isolate obtained from a fatal case of severe fever with thrombocytopenia syndrome (SFTS) in South Korea (*black dot*) compared with representative SFTS virus strains from China and Japan. The tree was constructed on the basis of the nucleic acid sequences of the RdRP genes by using the neighbor-joining method. Location, year of isolation, and GenBank accession numbers are indicated. Branch length of the tree shows the evolutionary distance. *Scale bar* indicates 2.0% sequence distance.
Data source: Kim KH, Yi J, Kim G, et al. Severe fever with thrombocytopenia syndrome, South Korea, 2012. *Emerg Infect Dis*. 2013;19:1892–1894.

genase. In one study of 285 patients from the Henan Province, investigators reported that a small number of patients experienced mental status alterations, ecchymosis, gastrointestinal hemorrhage, pulmonary hemorrhage, and disseminated intravascular coagulation.[295] Interestingly, many of these symptoms are similar to those of hemorrhagic fevers caused by other bunyaviruses such as Crimean-Congo hemorrhagic fever, hemorrhagic fever with renal syndrome, and Rift Valley fever. Although no indications of person-to-person transmission exist in the initial clusters of cases, several recent reports demonstrate person-to-person transmission of SFTSV among healthcare workers, family members, and mortuary workers.[211,297,298]

Epidemiological investigations during the initial cluster of cases showed that the majority of patients were farmers living in wooded and hilly areas and were working in the fields before the onset of disease.[296] Also, mosquitoes and ticks were commonly found in the patients' home environment. Thus, the role of arthropod vectors was highly suspected. Although no viral RNA was found in any of 5,900 mosquitoes tested, more than 5% of *Haemaphysalis longicornis* ticks collected from animals in the areas were the patients lived contained SFTSV RNA.[296] *H longicornis* is widely distributed in the Asia-Pacific region, including China, Korea, Japan, Australia, the Pacific Islands, and New Zealand.[299] SFTSV-specific antibodies and viral RNA have also been found in several domesticated animals in China.[300] In a sampling of more than 3,000 domesticated animals in Shandong Province, specific antibodies were detected in 69.5% of sheep, 60.5% of cattle, 37.9% of dogs, 3.1% of pigs, and 47.4% of chickens. SFTSV RNA was detected in all these animal hosts as well, albeit at a somewhat lower prevalence, ranging from 1.7% to 5.3%.[300] These findings demonstrate that natural infections of SFTSV occur in several domesticated animals in disease-endemic areas and that the virus has a wide host range. However, the role of domesticated animals in the circulation and transmission of SFTSV remains unclear.[300]

The disease has also been detected in Japan and South Korea, killing at least eight people in each country thus far.[301] This occurrence is perhaps not too surprising given that the range of the tick vector includes these countries and SFTSV was detected in *H longicornis* ticks collected during 2011 to 2012 in South Korea.[301] The strains isolated from South Korea were closely related to those from China, but were somewhat more distantly related to those from Japan (Figure 25-19),[301] which is consistent with the geographic distance between these countries and presumably reflects the greater evolutionary history between these viruses.

Heartland Virus

At about the same time that SFTSV was discovered in rural China (ie, 2009), a similar but distinct virus infected two men in rural Missouri in the United States. The men, one in his late 50s and the other in his late 60s who both lived on large farms in northwestern Missouri, independently presented to Heartland Regional Medical Center in Saint Joseph, Missouri, in early June 2009.[302] Both individuals had elevated temperatures exceeding 39°C, thrombocytopenia, and leukopenia. Other symptoms included elevation of the liver enzymes alanine aminotransferase and aspartate aminotransferase, nonbloody diarrhea, fatigue, and anorexia. The two men were hospitalized for 10 and 12 days, respectively, and both had short-term memory difficulty, which slowly improved over 4 to 6 weeks.[302] These symptoms sound remarkably similar to those of the Chinese patients suffering from SFTS.

Because all the specimens collected from these two individuals were negative for all the known pathogens, blood was sent to the CDC in Atlanta, Georgia, for further testing. Electron microscopy revealed viruses consistent with members of the family *Bunyaviridae*. Next-generation sequencing and phylogenetic analysis identified the viruses as novel members of the *Phlebovirus* genus. The authors named it the Heartland virus. The Heartland virus is most closely related to the SFTSV, but is clearly distinct because it shows amino acid differences in the viral polymerase and nucleoprotein of 27% and 38%, respectively.[302] This novel virus was also distinct from an uncharacterized bunyavirus called Lone Star virus, which was isolated in 1967 from an *A americanum* tick found on a woodchuck in western Kentucky. Comparison of the polymerase amino acid sequence showed that the Lone Star virus shared only 34% identity with the Heartland virus.[302]

Both patients infected with the Heartland virus in Missouri had reported being bitten by ticks 5 to 7 days before the onset of their illness. Given the similarity to SFTS, both in terms of the disease symptoms and the high percent identity of the virus to SFTSV, it was highly likely that Heartland virus was also tickborne. In 2012, investigators from the CDC collected and tested arthropods in areas of northwestern Missouri, including the farms of the two patients, to identify potential arthropod vectors for this new pathogen.[303] These investigators collected 56,428 ticks at 12 sites including both patients' farms. *A americanum* was the most frequently encountered tick and represented 97.5% of the collected ticks.[303] They grouped the ticks into pools by site, collection date, species, sex, and life stage. Ten pools composed of nymphs of *A americanum* were RT-PCR positive for the Heartland virus, and

eight pools yielded viable virus in cell culture. None of the 758 specimens (representing 12 species) of mosquitoes collected was positive for the virus.[303] Although more epidemiological and laboratory work is needed, these data strongly incriminate the *A americanum* tick as the vector of Heartland virus, at least in Missouri.

Ebola Epidemic in West Africa

On December 26, 2013, an 18-month-old boy from the village of Meliandou in the Guéckédou District of Guinea had experienced an illness characterized by fever, vomiting, and black stools. He died 2 days later.[304] Within a few weeks, his 3-year-old sister, mother, grandmother, and a nurse who had treated him all developed similar symptoms, and all died within a week.[305] Others in the surrounding area continued to get sick and die for the next several months. By the end of March 2014, the disease that had circulated in the southeastern corner of Guinea was identified as Ebola (for more detailed information on Ebola virus, see Chapter 23, Filoviruses).[306,307] By then, 111 clinically suspected cases and 79 deaths had occurred.

Full-genome sequencing of viral isolates from Guinea identified the virus as Zaire ebolavirus, but it represented a new genetic clade of virus, different from those circulating in the Democratic Republic of the Congo and Gabon. These data suggest that the virus may have been indigenous to Guinea and had not just "jumped" from another area where Ebola outbreaks have occurred. Ebola had not been seen in West Africa before, although serological evidence from patients with febrile illnesses had detected Ebola virus-specific antibodies in a subset of the population in Sierra Leone.[308] An epidemiological investigation found that the likely source of the virus that had infected the young boy was a colony of insectivorous free-tailed bats (*Mops condylurus*) that lived in a hollow tree in the village. Villagers reported that children played regularly in the tree and that a colony of bats lived in the tree.[309] Subsequent sequencing of a large number of Ebola virus isolates from the outbreak also indicated that the outbreak had likely started with a single transmission from the animal reservoir to human, followed by human-to-human transmission to sustain the outbreak.[310]

By March 30, 2014, cases had begun to be reported from the Foya district across the border in Liberia, and on May 24, Sierra Leone reported its first laboratory confirmed case.[311] The West African outbreak quickly became the largest outbreak of Ebola in recorded history, surpassing the previous record of 425 cases from the 2000 to 2001 outbreak in Uganda.[312] Previous outbreaks had occurred in relatively remote areas, limiting the ability of the virus to spread to large numbers of people. With the West African outbreak, however, cases were reported from the large capital cities of Monrovia, Liberia (~1 million inhabitants), Conakry, Guinea (~1.6 million inhabitants), and Freetown, Sierra Leone (~1.2 million inhabitants). Some models predicted that—if left unchecked—the total number of Ebola cases could exceed 1 million cases.[313] Spurred in part by worst-case scenario predictions such as these, international partners tried to bring the outbreak under control. US assistance focused on Liberia, and included the deployment of 3,000 soldiers to Liberia under Operation United Assistance to support logistics, train health workers, and build 17 Ebola treatment units.[314] British assistance efforts focused on Sierra Leone, and the French assisted the Guinean government.

The number of new cases per week peaked in late 2014 (September in Liberia, November in Sierra Leone, and December in Guinea).[315] During the height of the outbreak, Ebola virus-infected individuals were exported to Italy, Mali, Nigeria, Senegal, Spain, the United Kingdom, and the United States. Nigeria, Mali, and the United States also had limited local transmission of the virus originating from the imported cases. In the United States, a traveler from Liberia presented at a hospital in Dallas County, Texas, on September 25, 2014.[316] He presented with fever, abdominal pain, and headache, but was misdiagnosed with sinusitis and discharged. He returned to the hospital on September 28 with worsening symptoms, was admitted, and tested positive for Ebola virus infection on September 30. The patient died on October 8, but two nurses became infected while caring for him. The nurses both survived.

As of April 13, 2016, there were a total of 28,616 cases of Ebola (confirmed, probable, and suspected) with 11,310 deaths (40% case fatality rate).[317] Sierra Leone was the last country to have active cases of Ebola virus disease and was declared Ebola-free on March 17, 2016. An investigational vaccine for Ebola was tested in Guinea in 2015 and was shown to be 100% effective in preventing disease in contacts of confirmed Ebola cases.[318] The trial used a "ring vaccination" design in which contacts and contacts of contacts of confirmed Ebola patients were either immediately vaccinated or vaccinated after a delay of 21 days. After interim analysis of the data from the trial showed the high effectiveness of immediate vaccination, the data safety and monitoring board recommended that the delayed vaccination arm be dropped and all participants be offered immediate vaccination. An additional Ebola vaccine trial, the Sierra Leone Trial to Introduce a Vaccine Against Ebola (STRIVE) was performed in Sierra Leone between April and August 2015. Nearly 9,000 volunteers consisting of healthcare and frontline workers were vaccinated in a phase II/III trial. Due

to the decrease in incidence of Ebola virus disease during the duration of the STRIVE trial, efficacy data could not be obtained for the vaccine, but a subset of the participants was enrolled in safety and immunogenicity substudies. No adverse events were reported in the safety study, and the immunogenicity study is ongoing as of October 2016.

Viral Pathogen Discovery by High-Throughput DNA Sequencing

Traditional diagnosis of viral infections has required some foreknowledge of the viral pathogen that is suspected to be causing disease. For example, enzyme-linked immunosorbent assays require either recombinant antigens or antigens isolated from whole organisms. Real-time PCR assays require sequence information from the genomes of the organisms that are suspected to be present. Any novel agent that has a genome that is sufficiently divergent enough to alter the structure of an antigen or change a binding site for a real-time PCR probe may not be detected in these assays. The advent of microarrays that contain millions of short nucleic acid probes increases the likelihood that a pathogen can be detected. These arrays can screen for hundreds to thousands of pathogens in a single sample,[319,320] but still require the genome sequences of each pathogen to design probes. Novel or divergent pathogens may also escape identification by arrays.

When high-throughput massively parallel sequencing became available in 2005,[321] it was quickly realized that the hundreds of thousands to millions of sequencing reads obtained by this new form of sequencing could be used as a diagnostic tool. The first demonstration of this occurred in 2007 with the detection of Israeli acute paralysis virus in colonies of bees that suffered from colony collapse disorder (CCD).[319] RNA was isolated from bees from CCD and non-CCD bee colonies, amplified by random RT-PCR, and then sequenced on a 454 sequencer. Examination of the sequence reads identified large numbers of bacteria, fungi, and viruses, but only reads from Israeli acute paralysis virus seemed to correlate with the presence of CCD in a colony.

The first demonstrated use of massively parallel sequencing for the detection of a novel human pathogen occurred in 2008, when a novel arenavirus was detected in patients that had received visceral organ donations from a single donor.[322] The donor had died of cerebral hemorrhage 10 days after returning from a 3-month trip to the former Yugoslavia. His liver and kidneys were transplanted into three recipients, whose initial recoveries were unremarkable. Within 4 to 6 weeks of receiving the transplants, however, all three recipients died displaying various levels of encephalopathy. Tissues were collected from each recipient, and RNA was extracted for sequencing. After sequencing, 14 sequence reads out of more than 100,000 reads obtained showed amino-acid level similarity to lymphocytic choriomeningitis virus. The reads obtained by massively parallel sequencing were used to design PCR primers, and standard PCR and sequencing enabled the recovery of the full genome sequence of the novel virus. The virus was 72% to 87% identical to lymphocytic choriomeningitis virus at the nucleotide level, and 79% to 97% identical at the amino acid level. Despite these relatively high levels of identity to lymphocytic choriomeningitis virus, initial tests with diagnostic microarrays had failed to identify any candidate viruses as a cause of the infection.

Another example of the use of massively parallel sequencing to identify a novel pathogen was published in 2009.[323] In September 2008, a patient in critical condition with hemorrhagic fever was airlifted from Lusaka, Zambia to Standton, South Africa (a suburb of Johannesburg). Although the index patient was under care in the hospital, the disease spread to a paramedic who had been on the air ambulance flight from Lusaka, a nurse who attended to the index patient, and a hospital worker who had cleaned the index patient's room. A tertiary case of disease also spread to a nurse who attended to the sick paramedic. The first four patients died, and the fifth patient, who was treated with ribavirin, survived. Liver and skin sections were submitted to the CDC, where immunohistochemical staining with a monoclonal antibody broadly cross-reactive for Old World arenaviruses gave a positive result.[323] Subsequent RT-PCR with conserved arenavirus primers yielded partial sequences of the glycoprotein and nucleoprotein genes, indicating the presence of a novel arenavirus. Serum and tissue samples from some of the cases were submitted for massively parallel sequencing, yielding 5.6 kb of sequence from the novel arenavirus. The partial genome was used to design PCR amplicons, and those were used to recover the full genome of the virus. Analysis of the full genome indicated that the novel virus was phylogenetically distinct from previously known arenaviruses, and it was named Lujo virus after Lusaka and Johannesburg.

Both of the cases described previously used relatively low numbers of sequencing reads from a 454 sequencer (around 100,000 per sample) to identify genome fragments of the novel viruses. The fragments were then joined by PCR and conventional Sanger sequencing to obtain the full genome sequences. A third example demonstrated a different approach using the extremely high capacity of the Illumina HiSeq sequencer. In 2009, three people from a remote village in the Bas-Congo province of the Democratic

Republic of the Congo were stricken with a hemorrhagic fever of unknown origin.[324] The first case was a 15-year-old boy who presented to the hospital with malaise, epistaxis, conjunctival injection, gingival bleeding, hematemesis, and bloody diarrhea. The hemorrhagic symptoms had only started the previous day, and the patient died 2 days later from sudden circulatory collapse. The second case was a 13-year-old girl who presented with similar symptoms and died 3 days after onset of her disease. The final case was a 32-year-old male nurse who worked in the clinic where the original two patients had been seen. He was transferred to a regional hospital 2 days after the onset of disease, where he was treated with fluid resuscitation, blood transfusion, and antibiotics. He recovered spontaneously a few days later.

A serum sample that was taken from the nurse before his recovery tested negative by PCR for all viruses known to cause acute hemorrhagic fever in Africa. RNA was extracted from the serum and sequenced by 454, yielding a single read (of approximately 4,500) that had 41% identity to known rhabdoviruses. Attempts to recover more of the virus genome sequence by PCR were stymied by limited sample, so the sample was subjected to sequencing on the HiSeq. The HiSeq run yielded more than 140 million reads, with 30,000 of them mapping to the novel rhabdovirus. The large number of reads obtained allowed reconstruction of 98.2% of the genome and showed that the new virus, named Bas-Congo virus, was only distantly related to other rhabdoviruses.

Several other examples of the utility of next generation sequencing are applied to viral pathogen discovery, including Heartland virus and SFTSV mentioned previously in this chapter. However, several bottlenecks still remain to the widespread adoption of this technology in diagnostic settings. One of the most difficult aspects is simply analyzing the extremely large datasets that can be generated. In many cases, the overwhelming majority of sequence reads will be from the host (human) and trying to identify a pathogen is like looking for a needle in a haystack. Many groups are working on computer algorithms that can sort through the datasets to rapidly and correctly identify pathogen reads. No one algorithm has been successful, prompting the US Defense Threat Reduction Agency in 2013 to offer a $1 million prize through Innocentive.com to the team that can develop the most reliable and efficient algorithm. The prize ultimately went to a bioinformatics team at the University of Tübingen in Germany.

The unprecedented ability to detect all of the nucleic acid present in a diagnostic sample is a powerful tool for pathogen discovery, but it does have some pitfalls. An example of this pitfall was published in September 2013 when the genome of a highly divergent single stranded DNA virus was detected in samples from patients with chronic seronegative hepatitis and diarrhea of unknown etiology.[325] Deep sequencing of serum samples from patients with chronic hepatitis identified a virus that was related to both circoviruses and parvoviruses, and it was provisionally named parvovirus-like hybrid virus (PHV-1). Deep sequencing of diarrheal stool samples in a separate laboratory independently identified a virus that had 99% identity to PHV-1, which were named PHV-2. Both PHV-1 and PHV-2 were 99% identical to a virus named National Institutes of Health-Chonqing virus that had been identified in samples from Chinese seronegative hepatitis patients.[179] Suspicions about the frequency at which these viruses were being detected led to a reanalysis of the samples using different nucleic acid extraction reagents, and eventually led to the conclusion that all of the detections of these viruses were likely linked to commercial nucleic acid isolation spin-columns that had been used in all of the studies. PHV sequences were identified in metagenomic sequencing datasets from the coastal marine waters of North America, suggesting that PHV was linked to diatoms present in the marine waters that generate the silica matrix used in the commercial spin-columns.

As next-generation sequencing continues to increase in throughput and decreases in price, its utility for identifying novel viral pathogens will continue to increase. One can imagine a scenario in the not so distant future in which a clinician will be able to test for every pathogen present in a patient's sample without needing to pre-select tests for specific pathogens based on the patient's symptoms.

FUTURE THREATS

Genetically Engineered Organisms

Without human intervention, the natural world has produced innumerable microbial agents that continue to emerge as new or newly observed causes of disease. Human activity has also played a huge role in the emergence of many diseases, but this effect has—for the most part—been inadvertent, rather than deliberate. The spread of HIV, for example, can be attributed almost entirely to human behavior, and the same was true of the spread of smallpox. Historically, both microbial agents and the affected populations have tended toward change during the disease outbreaks. Examples from the human experience include the way in which diseases

such as smallpox and measles favored the survival of several generations of Europeans who were most resistant to these diseases, followed later by unchecked contagion and decimation of new populations when the same diseases were introduced to isolated islands and the New World.[326,327] A classic example of agent–host adaptation in animals was the intentional introduction of myxoma virus (an orthopoxvirus, reminiscent of smallpox in rabbits) into Australia to control or eliminate a scourge of rabbits. At first, mortalities were high in the Australian rabbits, but in time the rabbits acquired a degree of genetic resistance. In parallel, the circulating virus became diminished in its virulence, persisting and being shed over a longer period of time in infected rabbits.[328] For both rabbit and virus, natural selection blindly favored survival of the species. This natural order has been intentionally perturbed by humans, from the lifesaving selection of relatively benign forms of disease to use as vaccines against the most virulent forms (eg, variolation, or the classical adaptation of measles, mumps, and rubella vaccines) to the intentional selection of the most virulent disease agents in biological weapons programs (the latter finally stigmatized and outlawed as such in the Biological Weapons Convention Treaty). Other microbial perturbations have been unintended, such as the treatment-based selection of antibiotic resistant bacteria now widespread in hospitals.[329]

More recently, humankind has acquired the technical capacity to create microbial threats far more deadly than natural evolution could create or sustain. Genetic engineering, the intentional molecular manipulation of genes, has proven to have capacity for both good and ill. A few examples from open scientific literature will follow to illustrate the seriousness of the threat of genetically engineered microorganisms.

Antibiotic resistant strains of *B anthracis*, the causative agent of anthrax, have been derived not only by biological selection, but also more directly by genetic engineering.[330–332] Scientifically, the capacity to do so with any bacterial threat is unsurprising, but the implications are ominous. Similarly, for anyone moderately skilled in microbiology, it is obvious that otherwise harmless bacteria may be engineered to synthesize toxins made by unrelated lethal strains of bacteria. Buffering the threat, unauthorized conduct of most such experimentation has become not only difficult but also illegal—subject to fines and incarceration—in many countries including the United States. In the United States, federally funded research that may result in knowledge that could be used for nefarious purposes, so called dual use research of concern, is subject to review before initiation of research and also at the stage when the findings from such research are ready for submission for publication.

Viral genomes can now be easily manipulated in the laboratory and infectious viruses can be generated from plasmid DNA. The progression of this technology with human pathogens began some 20 years ago with the simpler viruses (positive sense, single-strand, small genomes) such as poliovirus,[333] alphaviruses,[205] and flaviviruses.[334] It has grown to include negative-strand viruses (eg, vesicular stomatitis virus, respiratory syncytial virus, Ebola virus) and segmented viruses (eg, influenza virus, Crimean-Congo hemorrhagic fever virus). Even the relatively large genome of vaccinia virus can be derived from DNA cloned into bacteria.[335] In a parenthetical observation that was alarming to some in its simplicity, the capacity to derive a human pathogenic virus (poliovirus) by chemical synthesis was demonstrated.[336] Even more controversial were the efforts to genetically resurrect the 1918 influenza virus that killed some 20 million persons before disappearing[152,337–339] and the proposals to genetically manipulate smallpox virus.[340] Experiments designed to create or improve vaccines, to understand interactions between virus and host, or to unveil some arcane mysteries of the viruses themselves have simultaneously proven the ease with which bioactive and sometimes harmful molecules may be inserted into viruses. Symbolizing this, a large body of work with recombinant poxviruses was widely considered to be entirely benign until it was reported that a mouse poxvirus (ectromelia virus) was rendered more virulent by its modification to co-express a molecule of the immune system (ie, interleukin-4).[341] This result was merely part of a progression of studies of similar design and outcome,[342] but its timing (2001) crystallized the potential problem.

Perhaps the most prominent example of dual use research of concern in recent years occurred in late 2011, when two independent research groups prepared to publish research studies in which mutations were introduced into highly pathogenic influenza H5N1 viruses that facilitated efficient transmission of the viruses in the ferret model.[188,191] The ensuing debate resulted in a self-imposed moratorium on such research by influenza scientists in the United States and internationally,[343] while a regulatory framework for the review of proposals for such gain-of-function studies was constructed.[344] As a result, research proposals for this type of study submitted for US federal funding are subject to additional layers of review. It is expected that other countries will follow suit, if they do not already have such a framework. For more detailed information, the reader is directed to a special issue of *Science* specifically devoted to the H5N1 gain-of-function research debate.[345]

Ultimately, the capacity to create deadly pathogens through genetic engineering is restrained in large part by technical knowledge and opportunity, and in the final analysis, by intent. That is, what is straightforward for skilled scientists is impossibly difficult for the untrained and unequipped. However, a determined person with the appropriate set of knowledge and skills may succeed in creating genetically engineered microorganisms. Unfortunately, such organisms could also be created by well-intentioned scientists who underestimate the unexpected consequences of their work.

What countermeasures and solutions exist? Laws and regulations to emphatically restrict accidental or intentional creation of new deadly organisms, or possession of the deadly agents already existing in nature, have been implemented in the United States (eg, 7 CFR Part 331, 9 CFR Part 121, and 42 CFR Part 73), but these bounds are difficult—if not impossible—to enforce internationally. Also helpful are the myriad coordination meetings and rehearsals for public health responses to pandemic natural threats such as smallpox or a pandemic influenza virus; in the case of the outbreak of a contagious genetically engineered microorganism, classical methods of epidemiology and quarantine would likely be exceedingly helpful. Also encouraging, the likely period of ignorance as to the nature and design of a newly emerged causative agent has been compressed as the newest technologies have been applied to both diagnostics and bioforensics. Less encouraging, development of specific medical countermeasures (vaccines, therapeutic drugs) for a previously unknown organism usually takes years. Some regard this as impetus to redirect greater funding toward discovery of generic methods of boosting innate immunity in persons to provide increased resistance to most or all infectious agents. A related approach is to target common cellular pathways used and shared by many unrelated agents, especially viruses. As with conventional agents, great localized harm could be done and widespread panic produced by genetically engineered microorganisms, even if medical countermeasures were nominally available.

Synthetic Biology

Genome synthesis is no longer limited to the realm of viral genomes. In 2008, Gibson et al published a paper describing the complete chemical synthesis of all 582,970 bases of the *Mycoplasma genitalium* genome.[346] The starting material for the synthesis was short oligonucleotides of the kind that can be purchased for $0.10 per base or less. These were gradually assembled into larger and larger pieces of DNA until the researchers cloned and maintained the complete genome in the form of a yeast artificial chromosome in *Saccharomyces cerevisiae*.

Following closely on the heels of this achievement, the same group published a second paper in 2010 detailing the complete chemical synthesis of the 1.08 megabase-pair genome of *M mycoides*.[347] This genome was synthesized in a manner similar to that described above, but the group went one step farther. The group transplanted the synthetic genome into the husk of a *M capricolum* cell from which the normal genome had been removed. The cellular materials left behind after removing the normal genome accepted the new, synthetic genome and kick-started replication of the novel bacterium called *M mycoides* JCVI-syn1.0 after the J. Craig Venter Institute where the work was performed. To prove that the new bacterium had the synthetic genome, the group had included watermarks encoded in the genome during synthesis. These watermarks used a cipher made of short nucleotide sequences to encode e-mail and web addresses, the names of the authors, and the following famous quotes:

- "To live, to err, to fall, to triumph, to recreate life out of life" (by James Joyce),
- "See things not as they are, but as they might be" (by Robert Oppenheimer), and
- "What I cannot create, I cannot understand" (by Richard Feynman).

SUMMARY

Emerging infectious diseases are among some of the most important future threats facing both military and civilian populations. These diseases are caused by a variety of infectious agents (ie, bacteria, viruses, fungi, and parasites), some of which are new to mankind, whereas others have been around for millennia, but are only newly recognized. Still others may be common commensals that have acquired virulence factors (eg, toxins) or antimicrobial resistance genes though natural or unnatural (ie, genetic engineering) means.

Despite many successes in disease control and prevention, infectious diseases remain the leading cause of death worldwide and the third leading cause of death in the United States. HIV/AIDS, which was first recognized in 1981, is the most dramatic example of an infectious disease that has rapidly emerged during the last 35 years. Despite the significant advances in treatment of HIV/AIDS, the pandemic will continue to put large numbers of people at risk for new and reemerging opportunistic infections. The rapid spread

of the West Nile virus across the United States after its introduction in 1999 and the increasing problem of antimicrobial resistance are other examples of the ability of microbes to emerge, adapt, and spread.

Future threats are difficult to predict but will likely include many of the topics covered in this chapter, including the following:

- increasingly complex challenges of foodborne and waterborne diseases,
- the threat of another influenza pandemic,
- emerging antibacterial and antiviral resistance, and
- the increasing incidence of zoonotic diseases.

Meeting these challenges will require a multidisciplinary approach using the expertise of physicians and veterinarians trained in public health, microbiologists, pathologists, ecologists, vector biologists, and military and civilian public health officials.

Emerging infectious diseases have been defined as those diseases which have been newly recognized or whose incidence has increased within the past 20 years. What new diseases will be encountered in the next 20 years? What role will the increasingly advanced fields of molecular biology, genomics, and synthetic biology play? Will infectious agents from the past be resurrected, as has been done with the 1918 influenza virus? Or will increasingly advanced bioterrorists or rogue nations create weapons though genetic engineering or synthetic biology? Only through increased knowledge gained from continued research in infectious diseases will we be able to meet the challenges of these future threats.

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Chapter 26

LABORATORY IDENTIFICATION OF THREATS

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SUMMARY

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INTRODUCTION

Medical diagnosis is the process by which clinicians attempt to deduce the cause of a particular disease or disorder in a sick individual. The goal of diagnosis is to assist in making correct medical decisions about the patient's treatment and prognosis. For infectious diseases, a variety of medical information is used to make a diagnosis including a physical examination, interview with the patient, medical history of the patient, and clinical findings as reported by laboratory tests. The focus of this chapter will center on laboratory tests used to diagnose biological threat agents. These tests represent a piece of the diagnostic puzzle and should not be used solely for diagnosis and treatment. Physical and clinical findings as well as medical history are critical to an accurate diagnosis, and integrating all available medical information as well as all available laboratory information reduces the chance for misdiagnosis. Diagnosis requires the synthesis of multiple pieces of information into a medical judgment that will be used to affect patient care; therefore, getting the right answer must always take priority over getting a quick answer.

The content of this review will focus on the current and future state of in vitro diagnostics, as defined by the Food and Drug Administration (FDA):

> Those reagents, instruments, and systems intended for use in diagnosis of disease or other conditions, including a determination of the state of health, in order to cure, mitigate, treat, or prevent disease or its sequelae. Such products are intended for use in the collection, preparation, and examination of specimens taken from the human body.[1]

For the purposes of this chapter, a biological threat is any infectious disease entity or biological toxin encountered, either through natural distribution or intentionally delivered by an opposing force to deter, delay, or defeat US or allied military forces. The majority of biological threats of military and public health relevance are contained in the Department of Health and Human Services (DHHS) select agents and toxins list of regulated biological select agents and toxins (Table 26-1). Many of these biological threats were part of offensive biological weapons programs at one time. As with other infectious disease assays, biological threat laboratory assays are subject to the same requirements and regulations to be regulatory compliant, meaning that the test can be used for patient care.

For laboratory assays, two critical elements must meet minimal standards to be considered regulatory compliant: (1) the laboratory performing the test must be qualified, and (2) the test being performed must be validated. The Clinical Laboratory Improvement Amendments (CLIAs), which were passed in 1988 (CLIA '88), regulate the quality of the clinical laboratory performing the testing, whereas section 210(h) of the Federal Food, Drug, and Cosmetic Act regulates in vitro diagnostic tests. As such, the FDA has oversight and regulatory authority to clear in vitro diagnostic tests (medical devices) for commercial sale and use. The combination of a CLIA-accredited laboratory performing an FDA-cleared diagnostic test results in a regulatory compliant diagnostic result that can be used for the patient's treatment and prognosis. For the Department of Defense (DoD), maintaining regulatory compliance in performing in vitro diagnostic tests in a deployed environment poses a significant challenge.

CLIA sets the standards for any laboratory that performs testing on human samples for use in disease diagnosis and treatment. The goal of CLIA was to improve the quality of any testing conducted for medical purposes, and DoD facilities are not exempt from the requirements. However, the DoD was allowed to develop a separate plan for ensuring quality and standards in diagnostic testing, the Clinical Laboratory Improvement Program (CLIP; DoD Instruction 6440.2).[2] CLIP is similar to CLIA with certain exceptions to meet military operational requirements. Both CLIA and CLIP govern the quality of the laboratory performing the diagnostic test and include standards for personnel, quality control, quality assurance, procedure manuals, proficiency testing, and inspections for adherence to the standards. CLIA and CLIP require laboratory registration to perform testing, and registrations are based on the level of test complexity that the laboratory is accredited to perform.

Minimal complexity tests (waived) are simple tests that do not require significant quality oversight, such as tests cleared by the FDA for home use. Moderate and high complexity tests require increased knowledge, training and experience, quality control, and interpretation and judgment. Moderate tests are typically more automated while high complexity tests require significant technical manipulation by personnel. The current FDA-cleared diagnostic system, the Joint Biological Agent Identification and Diagnostic System (JBAIDS), is a high complexity test, which can make it difficult to maintain a high complexity CLIA registration in a deployed setting.

Movement of laboratory diagnostic capabilities to forward locations is driving long-term goals for DoD medical diagnostic devices to be CLIA-waived devices. The Next Generation Diagnostic System (NGDS), the Biofire Defense FilmArray, will likely be a moderate complexity device, an incremental improvement over

TABLE 26-1

REGULATED BIOLOGICAL SELECT AGENTS AND TOXINS

HHS and USDA Select Agents and Toxins (7 CFR Part 331, 9 CFR Part 121, and 42 CFR Part 73)

HHS SELECT AGENTS AND TOXINS

- Abrin
- Botulinum neurotoxins*
- Botulinum neurotoxin-producing *Clostridium**
- Conotoxins
- *Coxiella burnetii*
- Crimean-Congo hemorrhagic fever virus
- Diacetoxyscirpenol
- Eastern equine encephalitis virus
- Ebola virus*
- *Francisella tularensis**
- Lassa fever virus
- Lujo virus
- Marburg virus*
- Monkeypox virus
- 1918 pandemic influenza virus
- Ricin
- *Rickettsia prowazekii*
- SARS-associated coronavirus (SARS-CoV)
- Saxitoxin
- South American hemorrhagic fever viruses:
  Chapare
  Guanarito
  Junin
  Machupo
  Sabia
- Staphylococcal enterotoxins A,B,C,D,&E subtypes
- T-2 toxin
- Tetrodotoxin
- Tick-borne encephalitis complex (flavi) viruses:
  Far Eastern subtype
  Siberian subtype
- Kyasanur Forest disease virus
- Omsk hemorrhagic fever virus
- Variola major virus (Smallpox virus)*
- Variola minor virus (Alastrim)*
- *Yersinia pestis**

OVERLAP SELECT AGENTS AND TOXINS

- *Bacillus anthracis**
- *Bacillus anthracis* Pasteur strain
- *Brucella abortus*
- *Brucella melitensis*
- *Brucella suis*
- *Burkholderia mallei**
- *Burkholderia pseudomallei**
- Hendra virus
- Nipah virus
- Rift Valley fever virus
- Venezuelan equine encephalitis virus

USDA SELECT AGENTS AND TOXINS

- African horse sickness virus
- African swine fever virus
- Avian influenza virus
- Classical swine fever virus
- Foot-and-mouth disease virus*
- Goat pox virus
- Lumpy skin disease virus
- *Mycoplasma capricolum*
- *Mycoplasma mycoides*
- Newcastle disease virus
- Peste des petits ruminants virus
- Rinderpest virus*
- Sheep pox virus
- Swine vesicular disease virus

USDA PLANT PROTECTION AND QUARANTINE SELECT AGENTS AND TOXINS

- *Peronosclerospora philippinensis*
- *Peronosclerospora sacchari*
- *Phoma glycinicola*
- *Ralstonia solanacearum*
- *Rathayibacter toxicus*
- *Sclerophthora rayssiae*
- *Synchytrium endobioticum*
- *Xanthomonas oryzae*

*Denotes Tier 1 agent.
CFR: Code of Federal Regulations; HHS: Health and Human Services; SARS: severe acute respiratory syndrome; USDA: US Department of Agriculture.
Data source: http://www.selectagents.gov/SelectAgentsandToxinsList.html (valid June 2016).

the currently deployed system. The system is already FDA cleared for several infectious disease diagnostics and received an Emergency Use Authorization (EUA) during the 2014 Ebola outbreak in West Africa. FDA-cleared assays for biological threats on the FilmArray will likely be available in 2017 and will replace the JBAIDS.

Although CLIA/CLIP requirements are based on test complexity, FDA requirements for clearance are based on the risk associated with the test, and risk is dependent on the potential harm associated with obtaining the wrong diagnostic result. The FDA classifies in vitro diagnostic tests as either class I (lowest risk), class II (moderate to high risk), or class III (highest

risk) medical devices. The currently fielded JBAIDS system is an FDA regulated class II device and currently resides in combat support hospitals within the US Army and within other medical treatment facilities for the Air Force and Navy. For the DoD, the challenge remains maintaining regulatory compliance in far forward operational settings.

The availability of FDA-cleared assays for biological threats remains somewhat limited. In vitro diagnostic tests for biological threats are orphan products, that is, there is not a large enough market to incentivize private industry to develop the tests because they are performed on an infrequent basis and, therefore, sales are limited. Consequently, most of the cleared diagnostic tests for biological threats have resulted from DoD acquisition programs. Currently cleared biological threat assays on the JBAIDS system include tests for *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, and *Coxiella burnetii*. The absence of useful and cleared infectious disease assays on JBAIDS hampers the utility of the system for clinicians. Future diagnostic devices for DoD would benefit from expanded capabilities for common infectious diseases of military relevance, not just those that are most likely to be used in a biological attack.

Although biological science technology continues to advance, it must be emphasized that the DoD currently fielded and regulatory compliant in vitro diagnostic tests for biological threat agents are based on nucleic acid amplification chemistry that is 30 years old and a rapid cycling polymerase chain reaction (PCR) platform that is more than 10 years old. With the NGDS acquisition program underway, the platform is likely to be only an incremental improvement over the currently fielded system. The two most likely improvements will be onboard integrated sample processing and a sample in/answer out analysis flow. In essence, the system is likely to be an automated nucleic acid amplification in vitro diagnostic platform. During this time, microarrays, mass spectrometry, and DNA sequencing have advanced significantly for the identification of infectious agents.[3] Yet none of these approaches has matured to the point of receiving FDA clearance for medical diagnostic use or offer the hope of a simplified test that can be performed in a deployed setting for biological threat agents.

Unlike technology, the ability of military laboratories to identify and confirm the presence of biological threats using regulatory compliant diagnostics matures at a much slower rate. This is not to discount the use of newer technologies by the DoD for environmental testing, vector surveillance, and population surveillance. These results can be used to make operational decisions, but they cannot be used for individual patient treatment, a concept often lost within the research and development community. The combination of using multiple diagnostic devices, multiple diagnostic markers, medical intelligence, medical acumen clinical signs and symptoms, and classical microbiology (Figure 26-1) still provides the most reliable approach for medical diagnosis of diseases to affect medical treatment or response after a biological threat attack.

To enhance readiness and ensure the availability of laboratory testing capabilities, military and civilian clinical laboratories are linked into a series of laboratory response networks. The Centers for Disease Control and Prevention (CDC) sponsors the preeminent Laboratory Response Network (LRN) for bioterrorism. More recently, the DoD also has established the Defense Laboratory Network to further enhance military readiness. Together, these efforts have improved the national preparedness for biological threat identification, but continuing research and development are needed to improve the speed, reliability, robustness, and user friendliness of the new diagnostic technologies. This chapter will review currently available and future capabilities for agent identification and diagnostic technologies to protect and sustain the health of military personnel.

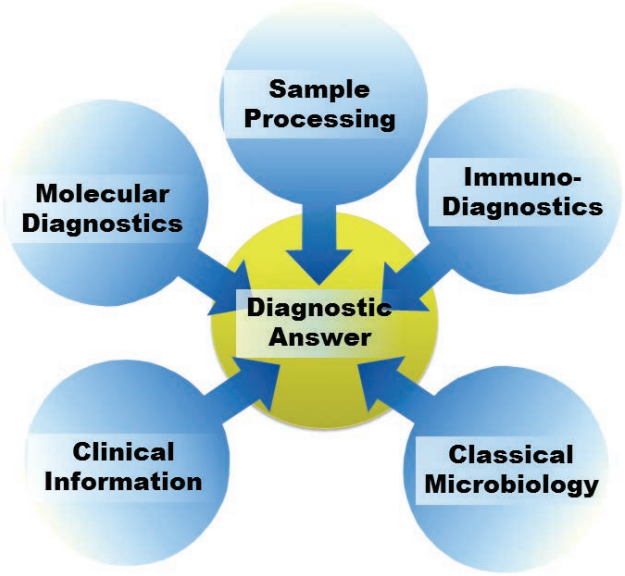


Figure 26-1. Orthogonal diagnostic testing uses an integrated testing strategy where more than one technology, technique, or biomarker is used to produce diagnostic results, which are then interpreted collectively. Although orthogonal diagnostic testing is a statistically independent approach, the combination of independent sensitivity and specificity values becomes highly valuable when combined. Orthogonal diagnostic testing improves the probability of reaching a "correct" result when the assays are less than 100% specific independently.

THE LABORATORY RESPONSE

Role of the Military Clinical and Field Laboratories

Military clinical and field laboratories play a critical role in the early recognition of biological threats. Intentionally delivered biological agents can also be used in bioterrorism scenarios to create terror or panic in civilian and military populations to achieve political, religious, or strategic goals. Although the principal function of military clinical laboratories is to provide data to support a clinical diagnosis, laboratory staff also provides subject matter expertise in theaters of operation on the handling and identification of hazardous microorganisms and biological toxins. In addition, these laboratories have a global view of disease in the theater and they play an important sentinel role by recognizing unique patterns of disease. Military field laboratory personnel may also evaluate environmental samples and veterinary medicine specimens as part of force health protection or a preventive medicine surveillance system in a theater of operations.[4] Military biological laboratory capabilities also exist to provide chemical, biological, radiological, and nuclear (CBRN) response, and elimination and remediation activities.[5]

Military Field Laboratories

Military field laboratories, which have many different configurations, are often incorporated into most of the services' basic deployable treatment facilities. If a complete medical treatment facility (MTF) is part of a deployment, its intrinsic medical laboratory assets can be used. However, a medical laboratory may not be available for short duration operations. In this case, medical laboratory support would be provided by a facility outside the area of operations.[6] A typical Army MTF in a theater of operations will have a limited initial microbiology capability even with the intrinsic laboratory component.

Following the removal of the microbiology capability from most Army medical treatment facilities under the 1994 Medical Reengineering Initiative, the capability has been restored with adding a microbiology augmentation set (Medical Materiel Set, laboratory [microbiology] augmentation UA N403 NSN 6545-01-505-2714 LIN M48987) and JBAIDS (UA 9409 NSN 6545-01-537-1100 LIN J00447). The N403 set contains necessary equipment and reagents to identify commonly encountered pathogenic bacteria. Susceptibility testing is not included. Although this medical set supports diagnostics of common bacterial infections, it does not contain an authoritative capability for identifying biological warfare agents. At the time of publication, the capability for biological threat agent detection is a mission primarily accomplished using JBAIDS. Specimens requiring more comprehensive analysis still require forwarding to the nearest reference or confirmatory laboratory, including the currently deployable assets for each service.

Army

The Area Medical Laboratory (AML) is a modular, task-organized, and corps-level asset providing comprehensive laboratory support to theater commanders.[5,7] The AML has transitioned from the original mission of testing primarily clinical specimens, with a capability for environmental samples (supporting force health protection) to being strictly an environmental sample testing lab. The AML can test for a broad range of biological, chemical, and radiological hazards. For biological agents, the laboratory uses a variety of rapid analytical methods, including molecular methods (such as real-time PCR), immunoassays (such as electrochemiluminescence [ECL] and enzyme-linked immunosorbent assay [ELISA]), and more advanced analyses involving bacterial culture, fatty acid profiling, and immunohistochemistry. The AML, which is the largest of the service deployable laboratories, can typically staff missions with a mix of microbiologists, biochemists, veterinary pathologists, and physicians. The AML maintains a degree of redundant equipment for long-term or split-base operations.

The 20th CBRNE Command (CBRNE—Chemical, Biological, Radiological, Nuclear, and Explosives), previously called the 20th Support Command CBRNE, fields a multitude of assets under a single operational headquarters. Its mission is to detect, identify, assess, render safe, dismantle, transfer, and dispose of unexploded ordnance, improvised explosive devices, and other CBRNE hazards, including biological warfare agents (see reference 14). The CBRNE Analytical & Remediation Activity Mobile Expeditionary Laboratory (CARA MEL), a unit within the 20th, provides high-throughput chemical, explosives, and biological sample analysis. It also has three mobile lab packages (a light mobile expeditionary lab, a heavy mobile expeditionary lab, and a chemical air monitoring system platform) that deploy to support weapons of mass destruction elimination and remediation efforts in forward deployed areas.

Navy

The Navy's forward deployable preventive medicine units are medium-sized mobile laboratories that use multiple rapid techniques (to include PCR and ELISA) for identifying biological warfare agents on the battlefield. The forward deployable preventive medicine units are modular and can analyze samples containing chemical and radiological hazards. These laboratories specialize in providing high confidence identification of biological threat agents in concentrated environmental samples, and they can identify endemic infectious disease in clinically relevant specimens.

Air Force

Air Force biological augmentation teams (unit type code FFBAT) and home station medical response laboratory biodetection teams use rapid analytical methods (such as real-time PCR) and immunological methods to screen environmental and clinical samples for threat agents.[8,9] The biological augmentation teams are small (two members), easily deployed, and typically housed in a separate facility designed to be collocated with preexisting or planned medical facilities. The units are capable of providing early warning to commanders about the potential presence of biological threat agents, typically in support of installation protection programs. The theater commander, in conjunction with the theater surgeon and nuclear, biological, and chemical officer, must decide which and how many of these laboratories are needed, based on factors such as the threat of a biological attack, the size of the theater, the number of detectors and sensitive sites in the theater, and the confidence level of results needed.

Defense Laboratory Network

The response to future CBRN threats will require an integrated military laboratory network that can respond with agility and competence. The logistical and technical burden of preparing for all possible health threats will be too great for the military clinical or field laboratories, which have limited space and weight restrictions. The most important role of these laboratories is to provide rapid and accurate laboratory support for medical diagnosis, rule out the most common threats, and alert the command about suspicious disease occurrences. The military Defense Laboratory Network consists of the front-line MTF clinical laboratories or deployed military laboratories backed by regional MTFs or military reference laboratories with access to more sophisticated diagnostic capabilities. The clinical laboratories in the regional medical centers or large medical activities are the gateways into the civilian LRN sponsored by the CDC.

At the top of the military response capability are research laboratories, such as the US Army Medical Research Institute of Infectious Diseases (USAMRIID; Fort Detrick, MD) and the Naval Medical Research Center (Silver Spring, MD). Other laboratories, such as the US Air Force Institute for Operational Health (Wright Patterson Air Force Base, OH) and the Naval Health Research Center (San Diego, CA), also provide reference laboratory services for a myriad of endemic infectious diseases. Military research laboratories have traditionally solved some of the most complex and difficult diagnostic problems, but they are not routinely organized to perform high-throughput clinical sample processing and evaluation. Sentinel laboratories are generally supported by the network's designated confirmatory laboratories, but they may communicate directly with national laboratories if necessary.

The network of military laboratories with connections to federal and state civilian response systems provides unparalleled depth and resources to the biological threat response (Figure 26-2). The Defense Laboratory Network is a standing member of the federal Integrated Consortium of Laboratory Networks (ICLN). The ICLN was established in 2005 under a memorandum of agreement signed by senior officials of federal agencies including the Departments of Agriculture, Defense, Commerce, Energy, Health and

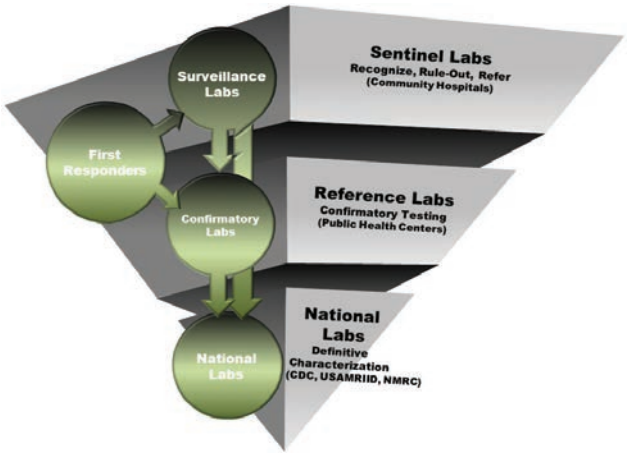


Figure 26-2. The network of military laboratories with connections to federal and state civilian response systems provides unparalleled depth and resources to the biological threat response. National Laboratory Response Network for Bioterrorism. CDC: Centers for Disease Control and Prevention; NMRC: Naval Medical Research Center; USAMRIID: US Army Medical Research Institute of Infectious Diseases.

Human Services, Homeland Security, Interior, Justice, and State, and the Environmental Protection Agency (https://www.icln.org/ valid February 2014). The ICLN was charged with promoting enhanced commonality and integration of network functions. Although the ICLN does not direct resources or operations, it does provide an environment for integrating network operations and strategies. The Department of Homeland Security (DHS) is charged with overall leadership and coordination.

Identification Levels

Rapid infectious disease diagnostics are not quantitative, not linked to traceable standards, and, overall, are not as well developed as other laboratory technologies. The inherent biological variability that exists between any two organisms (mammalian and microbial) complicates the ability to discern with absolute certainty the perpetrator of an infectious disease event. Laboratory tests for many infectious agents are not highly automated and still rely on decades-old technologies and techniques. Culture remains the gold standard for identifying organisms, but not all infectious disease agents can be grown in culture, or are difficult to culture in routine microbiology laboratories, making alternative methods necessary. These constraints significantly affect the confidence at which results on diagnostic or detection assays for infectious agents can be reported. It often goes unstated that the best that can be done in biology is that, with high confidence, what is incriminated as the infectious disease agent has high probability of being correct.

When microbiology culture capability is difficult or not available (eg, virus cultures in field laboratories), serological diagnosis (use of the antibody response) to the organism is still a useful method and sometimes the only way to discern some infections. The problem with both traditional culture and serodiagnosis is the time required to obtain results. Culture may take several days and serodiagnosis is constrained by the time required to mount an antibody response, which can exceed a week or more (Figure 26-3).

Within the past few decades molecular and immunodiagnostic technologies have been developed to improve the specificity and time to obtain diagnostic and detection information on infectious agents. Immunodiagnostic technologies are based on the use of antibodies as diagnostic reagents. Diagnostic and detection assays have been developed that can decrease detection times down to the range of minutes. Molecular diagnostics are based on the detection of specific nucleic acids characteristic of the infectious disease agent. Often the molecular diagnostic assay has to rely on the amplification of specific DNA sequences from extracted nucleic acids, DNA or RNA. Amplification techniques take tiny amounts of nucleic acid material and replicate them many times through enzymatic reactions, some that occur through cycles of heating and cooling. These techniques may bring more ambiguity on interpreting the results of the assays. Unlike cultured microbial agents, which can provide definitive results, immunodiagnostic and molecular diagnostic assays have various levels of false-positive and false-negative results. Discerning false-positive and false-negative results from true results becomes a risk management effort, aided by different levels of identification to express the degree of confidence associated with various testing methodologies.

Civilian

The CDC LRN uses two levels of identification: (1) presumptive and (2) confirmed.[10] In 1998,[11] following a demonstration that Iraq sponsored state activities involving production and use of biological weapons, President Clinton issued Presidential Decision Directive 62, Combating Terrorism, and assigned specific missions to federal departments and agencies. The

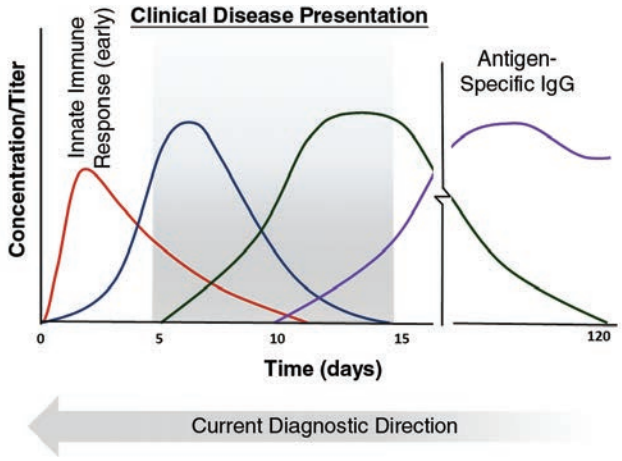


Figure 26-3. The typical infection and response time course begins with the initial pathogen encounter and leads to the formation and maintenance of active immunological memory (IgM and IgG) where serological detection is useful. Clinical disease, however, typically occurs around days 3 to 5 where detection of the infectious agent is possible. Often, by the time clinical disease is manifest, especially for the biological threat agents, clinical intervention to ensure survivability is not as effective as desired. To provide the most effective medical intervention on infectious agents, the closer to time 0 laboratory data is available, the more successful the outcome. IgG: immunoglobulin G; IgM: immunoglobulin M.

directive included a request to Congress to provide funding to the DHHS to support a renewed program of public health preparedness. In 1999, the LRN was established by the DHHS, the CDC in collaboration with founding partners, the Federal Bureau of Investigation, DoD, and the Association of Public Health Laboratories. The mission statement for the LRN is as follows:

> The LRN is a critical national security infrastructure asset that, with its partners, will develop, maintain, and strengthen an integrated domestic and international network of laboratories to respond rapidly to biological, chemical, and radiological threats and other high priority public health emergencies through training, rapid testing, timely notification and secure messaging of laboratory results.[11]

The LRN includes a biological network (LRN-B) and a chemical network. Identification of biological threat agents within LRN-B is—in part—based on the level of testing, which is based on the level of the laboratories where testing is performed. The different levels of laboratories within the LRN are sentinel, reference, and national laboratories (Figure 26-2). Sentinel laboratories represent the thousands of community based hospital laboratories that have direct contact with patients and may be the first to spot atypical infectious disease presentations. Sentinel laboratories do not actually confirm the presence of biological agents but rather are trained to recognize and appropriately handle biological agents that could potentially be extremely dangerous pathogens. Sentinel laboratories then refer these presumptive cultures to their closest LRN reference laboratory for more definitive testing. These reference laboratories perform standardized tests to detect, and typically confirm, the presence of biological agents that may represent a biological threat.

Reference laboratories, which are normally located within the respective state public health laboratories, perform reference-level tasks in biological safety level 3 (BSL-3) facilities.[12] Some LRN reference laboratories are located at county public health laboratories, animal health/veterinarian laboratories, military medical treatment facilities, and food safety laboratories. Public health directors can use LRN reference laboratory results to determine when a broad range of public health responses can be implemented. The CDC LRN protocols are currently limited to several bacterial agents, orthopoxviruses, and a couple of biologic toxins, and not all of the protocols have full confirmation methods for reference laboratory use (Table 26-2).

A recent programmatic change to the LRN system will subdivide the network configuration for the reference laboratories. The LRN reference laboratories will be broken down into limited (RL3), standard (RL2), and advanced levels (RL1). The standards for each level will be based in part on the minimum operational BSL, the core instrumentation and equipment available (including advanced platforms), and testing capabilities (number of agents and technologies). Limited laboratories (RL3) will typically do limited, specialized testing not meeting RL2 standards. Standard laboratories (RL2) will be typical state public health laboratories capable of the full agent testing capability on clinical and high risk environmental samples. Advanced laboratories (RL1), typically state public health laboratories in regional locations that cover a risk-based, priority population center (under the DHS Urban Area Security Initiative[13]), will be capable of additional testing capabilities using advanced detection and characterization methods such as mass spectrometry. The three national laboratories have unique resources to handle highly infectious agents (typically at BSL-3 and BSL-4[14]) and the ability to identify and characterize more agents, including BSL-4 viruses.

The CDC LRN, as a network, includes laboratories, secure communications, training, protocols, reagents, and proficiency testing. LRN member laboratories encompass federal laboratories (including laboratories at CDC, the US Department of Agriculture [USDA], the FDA, and other facilities run by federal agencies), state and local public health, military (DoD laboratories located both within the United States and abroad), food testing (FDA and USDA laboratories), environmental (water and other environmental samples), veterinary (USDA and state), and international laboratories (Canada, the United Kingdom, Australia, Mexico, and South Korea). As the LRN-B continues programmatic maturation, it will not only continue to address biological terrorism preparedness and response (national security and public health emergency preparedness), but also address emerging infectious disease preparedness and response (eg, newly emerging viruses such as Middle East respiratory syndrome coronavirus) and biosurveillance.

Military

Military identification levels differ from the civilian system in two specific aspects:

1. Current military doctrine includes four levels of identification (presumptive, field confirmation, theater level validation, and definitive) based, in part, on what level or what unit does the testing; and
2. Testing algorithms are based on the concept of testing for biological markers (biomarker) rather than culturing the specific agents.

TABLE 26-2

PRESUMPTIVE AND CONFIRMATION METHODS

Organism	Disease*	Presumptive†	Confirmatory (LRN)‡	Key Identity Markers	BSL-2	BSL-3
Bacillus anthracis	Anthrax	Nucleic acid amplification (PCR) Immunoassay (spore vs cell) - HHA or plate based	Culture and gamma phage with capsule or PCR of a culture with three assays	*B anthracis* is one of more than 260 different *Bacillus* spp. but is readily distinguishable from the others by the production of beta-hemolysin that is readily apparent on blood agar plates. *B anthracis* exists as both a vegetative cell and as an environmentally stable spore. *B anthracis* contains 2 plasmids, pXO1 and pXO2 that impart virulence characteristics and serve as diagnostic markers for both immunoassay and nucleic acid assays. Immunoassays will differ when testing for the vegetative cell or the spore. Whereas immunoassay and nucleic acid analysis can be used for diagnostic confirmation, culture is required for confirmation. Gram-positive rod; spore-forming; aerobic; nonmotile catalase positive; large, gray-white to white; nonhemolytic colonies on sheep blood agar plates.	Culture; PCR; immunoassay	Not needed

(**Table 26-2** *continues*)

Table 26-2 *continued*

Organism	Disease*	Presumptive†	Confirmatory (LRN)‡	Key Identity Markers	BSL-2	BSL-3
Brucella abortus *Brucella melitensis* *Brucella suis*	Brucellosis Brucellosis Brucellosis	Nucleic acid amplification (PCR) (not species specific) Immunoassay (not species specific) - HHA or plate based	Culture with biochemical testing	Depending on the taxonomy being used, brucellae contain 10 recognized species that include *B abortus*, *B melitensis*, and *B suis*, the most common and important human pathogens. Differentiating the human pathogenic species from the other brucellae, however, is not easy and requires several growth and biochemical determinations. Immunoassay and nucleic acid assays are currently not helpful in distinguishing the pathogens from the nonpathogens. Culture and biochemical testing are required for confirmation. Gram-negative coccobacilli or short rods; white, nonmotile, nonencapsulated, nonspore forming, slow-growing, nonhemolytic colonies on sheep blood agar plates; some species require enhanced CO_2 for growth.	Initial culture; PCR; immunoassay	Culture confirmation

(**Table 26-2** *continues*)

Table 26-2 *continued*

Organism	Disease*	Presumptive†	Confirmatory (LRN)‡	Key Identity Markers	BSL-2	BSL-3
Burkholderia mallei *Burkholderia pseudomallei*	Glanders Melioidosis	Nucleic acid amplification (PCR)	Culture with biochemical testing	*B mallei* and *B pseudomallei* are two of the 60 currently recognized species that include other human pathogens. As part of their environmental saprophytic lifestyle, the *Burkholderia* are complex organisms that are readily culturable, but often display colony morphology variations that confound routine microbiological analysis. Biochemical differentiation, including gentamicin and polymyxin susceptibility, determination of arginine dihydrolase and lysine decarboxylase, and arabinose fermentation are required for differentiation and confirmation. Gram-negative rod; oxidase-positive to variable, small, nonmotile, nonsporulating, nonencapsulated; primary isolation requires 48–72 h at 37°C; nonhemolytic, typically about 1 mm in width, white (turning yellow with age), *B pseudomallei* grows well on MacConkey agar, but *B mallei* does not.	Initial culture; PCR	Culture confirmation

(**Table 26-2** *continues*)

Table 26-2 *continued*

Organism	Disease*	Presumptive†	Confirmatory (LRN)‡	Key Identity Markers	BSL-2	BSL-3
Clostridium botulinum	Botulism A–E	Nucleic acid amplification (PCR) Immunoassay - HHA or plate based	Mouse testing	Gram-positive rod; spore-forming; obligate anaerobe catalase negative; lipase production on egg yolk agar; 150,000 Da protein toxin (types A–G); 2 subunits.	Initial culture; PCR; immunoassay; toxin-antitoxin neutralization test	Not needed
Clostridium perfringens	Epsilon toxin	Nucleic acid amplification (PCR)	Not in LRN	Gram-positive rod; spore-forming; obligate anaerobe catalase negative; 5 types (A-E), but only types B and D produce the epsilon toxin; on a blood agar plate produces double zone beta hemolysis.	Initial culture; PCR; immunoassay	Not needed
Coxiella burnetii	Q fever	Nucleic acid amplification (PCR) Immunoassay - HHA or plate based	Send to CDC	*C burnetii* is an obligate intracellular parasite that makes routine culture difficult. Culture in eggs or cells has previously been required so routine laboratory diagnostics are not common. Although highly infectious, *C burnetii* is typically not fatal and often serology is used for diagnosis. Direct fluorescent antibody and nucleic acid assays are often used for presumptive and confirmatory diagnostics.	PCR; immunoassay	Culture confirmation

(**Table 26-2** *continues*)

Table 26-2 *continued*

Organism	Disease*	Presumptive†	Confirmatory (LRN)‡	Key Identity Markers	BSL-2	BSL-3
Francisella tularensis	Tularemia	Nucleic acid amplification (PCR) Immunoassay - HHA or plate based	Culture with direct fluorescent antibody stain	*F tularensis* subspecies tularensis (type A) and *F tularensis* subspecies holarctica (type B) are the two most virulent strains of this expanding group of organisms. Until recently, *F tularensis* type A or B was restricted to the Northern Hemisphere where *F tularensis* type A or B is common in North America, but only *F tularensis* type B is typically found in Europe and Asia. *F tularensis* is relatively easy to grow and growth is required for confirmation, typically by the direct fluorescent antibody assay. Extremely small, pleomorphic, gram-negative coccobacilli; nonspore forming; facultative intracellular parasite; nonmotile; catalase positive opalescent smooth colonies on cysteine heart agar.	Initial culture; PCR; immunoassay	Culture confirmation
Rickettsia prowazekii	Louse-borne typhus, *Typhus exanthematicus*	JBAIDS nucleic acid amplification (PCR)	Not in LRN	Gram-negative, obligate intracellular parasitic, aerobic bacteria.	PCR	Culture confirmation
Rickettsia rickettsii	Spotted fever	Nucleic acid amplification (PCR) Immunoassay - HHA or plate based				

(**Table 26-2** *continues*)

Table 26-2 *continued*

Organism	Disease*	Presumptive†	Confirmatory (LRN)‡	Key Identity Markers	BSL-2	BSL-3
Staphylococcus aureus	Enterotoxins A and B (SEA & SEB)	Immunoassay - HHA or plate based	Not in LRN	Gram-positive, cocci; facultative anaerobic, large round white to yellow, beta-hemolytic colonies on sheep blood agar; characteristic "grape-cluster" on Gram stain; catalase and coagulase-positive; multiple toxins depend on strain.	Initial culture; immunoassay	Not needed
Yersinia pestis	Plague	Nucleic acid amplification (PCR) Immunoassay - HHA or plate based	Culture with phage testing	*Y pestis* belongs to a smaller group of organisms, but is much more difficult to correctly identify. *Y pestis* has several plasmids that confer various virulence traits and are useful diagnostic assay targets, but the plasmids are promiscuous and can be found in non-*Y pestis* causing the potential for false-positive assays. Capsule (F1) is a good marker for the diagnosis of *Y pestis*, but does not get produced at the optimal growth temperature for *Y pestis* (28°C). Instead, it is produced at 35°–37°C, making this marker less reliable for environmental *Y pestis* detection. Immunoassay and nucleic acid assays are available for diagnostics, but confirmation of *Y pestis* is done using phage on cultural growth. Gram-negative coccobacilli often pleomorphic; nonspore forming; facultative anaerobe; nonmotile beaten copper colonies (MacConkey agar).	Initial culture; PCR; immunoassay	Culture confirmation

(**Table 26-2** *continues*)

Table 26-2 *continued*

Organism	Disease*	Presumptive†	Confirmatory (LRN)‡	Key Identity Markers	BSL-2	BSL-3
Crimean-Congo hemorrhagic fever virus/bunyaviruses	Viral hemorrhagic fevers	Nucleic acid amplification (PCR)	Not in LRN	Single negative-stranded, tripartite genomes (large [RNA-polymerase], medium [glycoproteins], small [nucleocapsid protein]) exist in a helical/pseudo-circular structure; enveloped RNA viruses.	PCR	Culture confirmation/ BSL-4
Ebola, Marburg virus/filoviridae viruses	Viral hemorrhagic fevers	Nucleic acid amplification (PCR)	Not in LRN	Linear, negative-sense single-stranded RNA virus; enveloped; filamentous or pleomorphic, with extensive branching, or U-shaped, 6-shaped, or circular forms; limited cytopathic effect in Vero cells.	PCR	BSL-4
Lassa/ arenaviruses	Viral hemorrhagic fevers	Nucleic acid amplification (PCR)	Not in LRN	Two single-stranded RNA segments ambisense RNA virus; beaded nucleocapsid, spherical with glycoprotein spikes.	PCR	BSL-4
Variola major	Smallpox	Nucleic acid amplification (PCR) Immunoassay - HHA or plate based	Send to CDC	Large double-stranded DNA virus; enveloped, brick-shaped morphology; Guarnieri bodies (virus inclusions) under light microscopy.	PCR; immunoassay	BSL-4 (CDC ONLY)
Venezuelan equine encephalitis virus/ alpha viruses	Viral encephalitic disease	Nucleic acid amplification (PCR) Immunoassay - HHA or plate based	Not in LRN	Linear positive-sense single-stranded RNA virus; enveloped, spherical virions with distinct glycoprotein spikes; cytopathic effect in Vero cells.	PCR	Culture confirmation
Yellow fever virus/flaviviruses	Viral encephalitic disease	Nucleic acid amplification (PCR)	Not in LRN	Linear positive-sense single-stranded RNA virus; enveloped, icosahedral nucleocapsid; cytopathic effect in Vero cells.	PCR	Culture confirmation

(**Table 26-2** *continues*)

Table 26-2 *continued*

Organism	Disease*	Presumptive†	Confirmatory (LRN)‡	Key Identity Markers	BSL-2	BSL-3
Ricin toxin	Ricin intoxication	Nucleic acid amplification (PCR) Immunoassay - HHA or plate based	Send to CDC	60,000–65,000 Da protein toxin; 2 subunits castor bean origin.	PCR; immunoassay	Not needed

*Disease refers to the disease state induced by the agent or the disease-causing entity of the agent.
†Presumptive refers to typical diagnostic assay techniques used for reporting the presumptive evidence of a disease-causing agent.
‡Confirmatory (LRN) refers to the diagnostic assay techniques used for reporting the confirmed evidence of a disease-causing agent being present.
BSL: biological safety level
CDC: Centers for Disease Control and Prevention
HHA: hand held immunoassay
JBAIDS: Joint Biological Agent Identification and Diagnostic System
LRN: Laboratory Response Network
PCR: polymerase chain reaction
RNA: ribonucleic acid
SEA: Staphylococcal enterotoxin A
SEB: Staphylococcal enterotoxin B

The military concept of testing for biomarkers follows the logic that if the biomarker is present, then the agent of interest is also present. Some complications exist with using biomarkers. One problem is defining a biomarker. *Multiservice Tactics, Techniques, and Procedures (MTTP) for Biological Surveillance Editor*[15] provided a definition, but that document was replaced by Army Techniques Publication 3-11.37[7] in 2013 and the definition was lost. The current doctrinal revision to Army Techniques Publication ATP 4-02.7/MCRP 4-11.1F/NTTP 4-02.7/AFTTP 3-42.3 15 March 2016, *MTTP for Health Services Support in a CBRN Environment*, reestablishes the definition as:

> A biomarker refers to a detectable/measurable substance that is correlated with the presence of a BW [biological warfare] agent (bacteria, virus, or toxin). Biomarkers should be unique to the biological agent, often associated with virulence, and can be independent of the biological agent's viability/infectivity/functionality.

The types of biomarkers listed included nucleic acid sequences, antigens or toxins for immunological methods, growth properties (as demonstrated on biochemical tests or selective media), and microscopic characteristics. The revised doctrinal definition will help guide correct application in the absence of specific details. Another scientific concern with the use of biomarkers is that some biomarkers are present due to nonthreat infectious agents inducing similar biomarker profiles to threat agents. Although these results are considered false-positives for biothreats, induction of disease specific profiles still indicates infection and therefore can remain useful in the overall determination of the etiologic agent.

Biomarkers also do not necessarily reflect viability of the infectious agent. Although the simple presence or absence of an agent can be important, determination of viability may be a significant component, especially in nonclinical samples where the biomarker could be simply background flora. When laboratories rely on biomarkers in lieu of culture, the ability to determine other critical information is often lost, such as antimicrobial resistance, epidemiological strain typing, or legal evidence for forensic science and attribution purposes. Concentrating on biomarkers may lead to a myopic result that limits the full understanding of medical implications for an incident or outbreak.

The military identification levels are well defined in doctrine as follows[5,7] (Figure 26-4). Presumptive identification of a biological threat agent is achieved by the detection of a biological marker using a single test methodology (eg, hand held assay [HHA]). Presumptive identification uses technologies with limited specificity and sensitivity by general purpose forces in a field environment to determine the presence of a biological hazard with a low level of confidence but with a degree of certainty necessary to support immediate tactical decisions. Since identification at this level is based on specific technologies, it is limited to the assays deployed and cannot detect or identify new or emerging infectious disease agents for which the technologies assays are not available.

Field confirmatory identification is achieved when two or more independent technologies confirm the identification of a biological agent. This may be an immunoassay (eg, HHA, ECL, ELISA, nucleic acid amplification result, and/or culture growth/microscopy). According to doctrine, a single result from JBAIDS can be used as a field confirmatory identification. A genomic biomarker must be included. Field confirmatory identification uses technologies with increased specificity and sensitivity, by technical forces in a field environment, to identify the presence of biological agents with a moderate level of confidence and a degree of certainty necessary to support follow-on tactical decisions. Depending on the technologies deployed (eg, culture), some limited ability exists to detect or identify infectious disease agents beyond the limits of deployed assays.

Theater validation is achieved using devices, materials, or technologies that detect biomarkers using two or more independent biomarker results (ie, one biomarker is detected by two or more independent methodologies or more than one biomarker is detected by a single methodology). Examples are: (1) hand held immunological assay plus nucleic acid amplification or (2) nucleic acid amplification using two different biomarkers (eg, gene targets). Theater validation identification uses multiple independent, established protocols and technologies by scientific experts in a controlled environment of a fixed or mobile/transportable laboratory to characterize biological materials with a high level of confidence and the degree of certainty necessary to support operational level decisions. After a preventive medicine detachment, a combat support hospital or CBRN reconnaissance assets identify a biological/clinical specimen as a biological threat agent, the specimen is sent by courier to those specialized laboratories/teams with advanced microbiological capabilities and highly skilled medical personnel. These could include laboratories/teams such as an AML, 20th CBRNE CARA MEL, the US Air Force biological augmentation team, or the US Navy forward

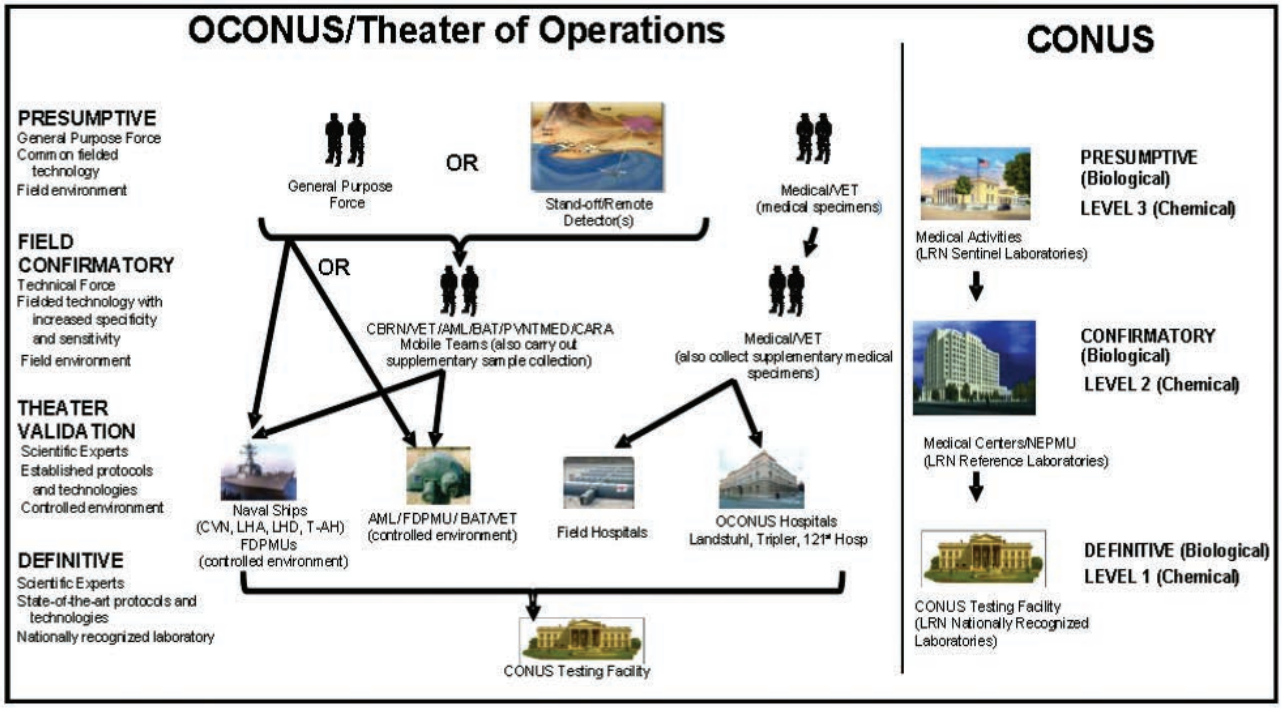


Figure 26-4. Military identification levels correspond with both the technology used and the facility doing the laboratory analysis. Although there is some correspondence to the civilian Centers for Disease Control and Prevention's Laboratory Response Network system, the military system has significantly unique aspects.
AML: area medical laboratory; BAT: biological augmentation team; CARA: CBRNE analytical remediation activity; CBRN: chemical, biological, radiological, and nuclear; CBRNE: chemical, biological, radiological, nuclear, and explosives; CONUS: continental United States; CVN: aircraft carrier, nuclear; FDPMU: forward deployed preventive medicine unit; Hosp: hospital; LHA: amphibious assault ship (general purpose); LHD: amphibious assault ship (multipurpose); LRN: Laboratory Response Network; NEPMU: Navy environmental and preventive medicine unit; OCONUS: outside the continental United States; PVNTMED: preventive medicine; T-AH: hospital ship; VET: veterinary

deployable preventive medicine unit when available in the operational area. Although the units listed here have the potential to produce theater validation level results, they may not inherently have that capability deployed in all circumstances.

The theater validation laboratories must implement a quality assurance program, preferably with independent audits, proficiency testing, scientist level data review, document control, demonstration of procedure traceability, some level of electronic sample management, documentation of personnel training, and accreditation (if available). These laboratories would typically conduct initial field confirmatory analysis (quick report) followed by theater validation (more testing and time). If these specialized laboratories/teams are unavailable, biological specimens that are presumptively positive for a biological threat agent will have to be forwarded to the nearest reference laboratory, even if this is in the continental United States (CONUS).

Definitive identification is the correlation of a biological agent to a known substance, or in the case where the substance is previously unknown, the substance is type classified and analyzed. Definitive identification is the use of multiple state-of-the-art, independent, established protocols and technologies by scientific experts in a nationally recognized laboratory to determine the unambiguous identity of a biological agent with the highest level of confidence and degree of certainty necessary to support strategic level decisions. It also supports the initiation of attribution to implicate or point to the source of the identified material. In all cases a definitive identification occurs at a US-based and sanctioned reference laboratory specifically equipped to perform detailed analysis on the type of suspect material to be identified. Definitive identification typically includes the ability to propagate the biological agent so that there is sufficient material available for analysis by the multiple methods and protocols, and the ability to look at strains by epidemiological methods, but also so material is available to initiate attribution analysis. Definitive identification is performed using the highest level quality assurance measures in a controlled laboratory. Definitive identification or "confirmation" testing is performed at sanctioned reference laboratories, including reference laboratories of the CDC LRN as appropriate. Specific LRN protocols and reagents are proprietary, but any definitive identification or confirmation typically follows a well-established scheme, including the use of well characterized reagents by well-practiced personnel.

Like biomarkers, there are also inherent problems with the application and details involved in the identification levels that need to be understood to correctly apply the inherent concepts contained within the definitions and an appropriate application of the term confirmed. In one definition of confirmation, it states "the occurrence of two or more indicators corresponding with one another and thereby corroborating the predicted outcome." Confirmation of an identification of a biological agent, however, often needs to be grounded in more information, especially given the consequences of an incorrect identification to both the military member as well as the military operation being conducted. In addition, identification of a biological agent based on nonmetabolic methods, in the absence of morbidity or mortality, always presents the possibility that the identification is detecting inactive materials.[16]

Biological materials, microbes and toxins, are fragile compared to nuclear or chemical agents. They can be inactivated during the course of dispersal (especially dissemination from munitions), through natural biocidal activity (sunlight both desiccates as well as inactivates through ultraviolet irradiation), ineffective weaponization processes, or myriad physical or chemical activities. The confidence in an identification of a biological attack is also affected by how it has been detected.[7,8] Doctrinally, low, medium, and high confidence are part of the identification levels, yet the level of confidence an assay provides is also governed by factors that include the scientific quality and accuracy of the test methods, the target or purpose of the assay(s), experience and knowledge of testing personnel, and the environment in which the lab is operating.[8] Detection by one biological detector system has a lower confidence level than if two detectors have made the detection. Theater validation identification (including two biomarkers) endorses and bolsters those automated detections, but confirmation should still be viewed with a level of suspicion resulting from inherent biological diversity. Until a full characterization of the agent can be undertaken, the term confirmed should be used with some level of reservation; and military commanders, responsible for both the mission and the welfare of service members, should proceed with the realization of the ambiguous nature that biological threats present.

Allies

US allies, including members of the North Atlantic Treaty Organization, have different doctrinal identification levels. Before 1995, the North Atlantic Treaty Organization recognized the need for common approaches for sampling and identification of biological and chemical warfare agents. Within its doctrine, three levels of identification also exist[16]:

1. Provisional identification: A biological agent may be considered provisionally identified when one of three criteria is met (presence of a unique antigen, presence of a unique nucleic acid sequence, or positive culture or multi-metabolic assay);
2. Confirmed identification: The identification of a biological agent is confirmed when any two of the three criteria for provisional identification have been met in the presence of authentic reference standards (positive and negative controls) under identical experimental conditions; and
3. Unambiguous identification: The unambiguous identification of a biological agent provides the highest level of certainty required for the development of strategic and political positions. Confirmed identification becomes unambiguous under four criteria: (1) positive response is obtained by a genetic identification method; (2) positive response is obtained by an immunological method; (3) positive match is obtained by in vitro culture or multimetabolic assay; and (4) the disease properties of the microbial agent are confirmed in an accepted animal model.

IDENTIFICATION APPROACHES

Specimen Collection and Processing

Clinical specimens can be divided into three different categories based on the ability to affect the disease course: (1) early postexposure, (2) clinical, and (3) convalescent/terminal/postmortem.[5,17] Common specimens for biological warfare agents are similar to those collected for diagnosis of any infectious disease and typically correspond to clinical manifestations (Table 26-3). Specimens often include swabs, induced respiratory secretions, blood cultures, serum, sputum, urine, stool, skin scrapings, lesion aspirates, and biopsy materials.[5,18] Nasal and facial swab samples should not be used for making decisions about individual medical care; however, they could support the rapid identification of a biological threat (postattack) and help direct force health protection efforts.[19,20] Baseline serum samples (presymptomatic) should be collected on all potentially exposed personnel after an overt attack. These samples will help to both define the forces exposed but could also provide diagnostic information in the event that nontraditional agents are being used.

In cases of sudden or suspicious deaths, autopsy samples should be taken. Specimens and cultures containing possible highly infectious agents should be handled in accordance with established biosafety precautions. Specimens should be sent rapidly (within 24 hours) on wet ice (2°C–8°C) to an analytical laboratory capable of handling them. Blood cultures should be collected before the administration of antibiotics. If necessary, the blood cultures should be shipped to the laboratory within 24 hours at room temperature (21°C–23°C). Overseas laboratories should not attempt to ship clinical specimens to CONUS reference laboratories using only wet ice unless the provisions for reicing the samples are made with the carrier. Shipments requiring more than 24 hours should be frozen on dry ice or liquid nitrogen if possible. Specific shipping guidance should be obtained from the supporting laboratory before shipment. Specimens should not be treated with permanent fixatives (ie, formalin or formaldehyde) unless that is the only way to ensure sample stability. Storage and shipping of samples at –20°C to 25°C is contraindicated.

Environmental samples, while not patient specific, are often highly useful to medical decision making. These samples include several different categories of materials such as buffers and filters from air sampling devices, powders, soil and vegetation, animals (including rodents and insects as potential vectors), food samples from both fresh and packaged materials if ingestion is suspected, and nearly everything else that is not a clinical sample. These samples, when taken before any overt disease onset, can help identify a causative agent and potentially lead to prophylactic treatment. Nonclinical samples represent the biggest challenge in the detection of biological agents because of the vast repertoire of sample types and microorganisms in the environment that cause false-positive and false-negative detection reactions in many laboratory assays.

A substantial amount of guidance exists—both military specific[5,16] and general[21]—so details of taking and processing of environmental samples is beyond the scope of this chapter. Environmental samples will contain myriad physical and chemical agents that can potentially interfere with detection technologies and cause false negative results. Environmental samples include samples that are both highly stable as well as samples that will degrade with time similar to clinical samples. Guidelines for the submission of environmental samples are not as well detailed as those for clinical samples. In general, environmental samples should be maintained at nearly the same state as when they were collected. Dry samples should be kept dry, moist or wet samples should be preserved from desiccation, and

TABLE 26-3

SPECIMEN COLLECTION FOR SELECT BIOLOGICAL WARFARE AGENTS

		Postexposure†		Clinical		Convalescent/Terminal/Postmortem‡	
Organism	**Incubation Period***	**Time**	**Samples**	**Time**	**Samples**	**Time**	**Samples**
Bacillus anthracis	1–6 d; 3 d	0–72 h	Nasal and throat swabs, and induced respiratory secretions	48–72 h	Serum for toxin assays; whole blood (blood cultures) and tissue smears for direct fluorescent antibody§	72 h–28 d	Serum for toxin assays; whole blood (blood cultures)
Brucella	5–60 d; 5 d	0–36 h	Nasal and throat swabs, and induced respiratory; *note*: notify laboratory for extended culture incubation protocol	72–168 h	Whole blood (blood cultures); *note*: notify laboratory for extended blood culture incubation protocol	7–28 d	Serum for immunoassays; whole blood (blood cultures); *note*: notify laboratory for extended blood culture incubation protocol
Burkholderia pseudomallei/ mallei	1–21 d; 3 d	0–48 h	Nasal and throat swabs, and induced respiratory secretions	24–96 h	Serum for capsular polysaccharide assays; whole blood (blood cultures)	7–28 d	Serum for capsular polysaccharide assays; whole blood (blood cultures)
Clostridium botulinum/ botulinum toxins A/B/E		0–24 h	Nasal and throat swabs, and induced respiratory secretions for toxin detection	24–72 h	Blood or serum for toxin detection	7–28 d	None; serum for IgM and IgG not really valid
Coxiella burnetii	7–41 d	0–72 h	Nasal and throat swabs, and induced respiratory secretions (egg, tissue culture, or axenic media)	3–14 d	Whole blood (blood cultures) and direct molecular detection¥	14–60 d	Serum for IgA, IgM, and IgG
Encephalitic viruses/ alpha viruses/VEE/etc	2–6 d	0–24 h	Nasal and throat swabs, and induced respiratory secretions	24–72 h	Throat swabs up to 5 days, then cerebro-spinal fluid and serum	6–21 d	Serum for IgM and IgG
Francisella tularensis	1–21 d; 3 d	0–24 h	Nasal and throat swabs, and induced respiratory secretions	24–72 h	Whole blood (blood cultures); direct fluorescent antibody§	6–21 d	Serum for IgM and IgG

(**Table 26-3** *continues*)

Table 26-3 *continued*

Hemorrhagic fever viruses/Ebola/Marburg/ Dengue/etc	4–21 d	0–24 h	Nasal and throat swabs, and induced respiratory secretions	2–5 d	Serum	6–21 d	Serum or for IgM and IgG
Ricin	18–24 h	0–24 h	Nasal and throat swabs, and induced respiratory secretions	24–48 h	Serum/plasma for toxin assays; urine for ricinine is questionable	6–21 d	Serum for IgM and IgG
Staphylococcal enterotoxins A/B/C	3–12 h	0–4 h	Nasal and throat swabs, and induced respiratory secretions for toxin detection	2–6 h	Blood or serum		None; serum for IgM and IgG not really valid
Vesicular and pustular rash illnesses/*Orthopox* (*Variola*)	7–17 d	0–72 h	Nasal and throat swabs, and induced respiratory secretions	2–5 d	Serum and lesions/ scrapings for microscopy and viral culture	6–21 d	Lesions/scrapings for microscopy, and viral culture; serum for IgM and IgG
Yersinia pestis	1–7 d; 2 d	0–72 h	Nasal and throat swabs, and induced respiratory secretions	24–72 h	Whole blood (blood cultures); direct fluorescent antibody[§]	7–10 d	Whole blood (blood cultures); serum for IgM and IgG*; typical period; initial presentation of a high-dose exposure; dependent on dose; aerosol route

*Typical period; initial presentation of a high-dose exposure; dependent on dose; aerosol route.
[†]Rapid molecular and immunoassays can be done, but none are FDA cleared for patient treatment.
[‡]Serology and other tests may not be FDA cleared for patient treatment, but convalescent/terminal/postmortem testing is rarely used to influence direct patient treatments.
[§]Direct fluorescent antibody tests are not FDA approved, but accepted if done as a laboratory-developed test and validation data available.
[¥]FDA approved for direct patient treatment.
IgA: immunoglobulin A; IgG: immunoglobulin G; IgM: immunoglobulin M; VEE:Venezuelan equine encephalitis

cold samples should be kept cool. One especially critical requirement for any environmental sample is the initiation and maintenance of chain of custody documentation,[7,16,22] from the sample collection through to the analysis laboratory. Again, like shipping clinical samples, guidance should be obtained from the supporting laboratory before shipment.

A multitude of international, domestic, and commercial regulations mandate the proper packing and documentation (including labeling) of biological materials (Table 26-4). Biological samples, infectious agents, and biological select agents and toxins all represent some level of dangerous goods that need special handling to protect the public, airline workers, couriers, and other persons who work for commercial shippers and who handle the dangerous goods within the shipping process. In addition, proper packing and shipping of dangerous goods reduces the exposure of the shipper to the risks of criminal and civil liabilities associated with shipping dangerous goods, particularly infectious substances. Each of the regulations deals with specific shipping requirements, but in general, all define an infectious substance as a material known or reasonably expected to contain a pathogen (a microorganism that can cause disease in humans or animals). Universal examples of pathogens include bacteria, viruses, fungi, and other infectious agents. An infectious substance is assigned to one of the following three potential categories:

1. Category A: An infectious substance transported in a form capable of causing permanent disability or life-threatening or fatal disease in otherwise healthy humans or animals when exposure occurs. Category A infectious substances are assigned the identification number UN 2814 or UN 2900, based on the known medical history or symptoms of the source patient or animal, endemic local conditions, or professional judgment concerning the individual circumstances of the source human or animal.
2. Category B: An infectious substance that does not meet the criteria for inclusion in Category A. Category B infectious substances bear the shipping term "Biological substance, Category B" and are assigned the identification number UN 3373.
3. Toxins from plant, animal, or bacterial sources that do not contain an infectious substance and are not contained in an infectious substance may be considered for classification as toxic substances; and they are assigned the identification number UN 3172.

In addition, other requirements may exist, including requirements for dry ice (dry ice is classified by the Department of Transportation and the International Air Transport Association as a "miscellaneous" hazard, class 9). The International Air Transport Association manual, *Dangerous Goods Regulations,* is the leading guide to shipping dangerous goods, including infectious agents by air, which generally includes most shipments from CONUS and outside of the continental United States (OCONUS). *Dangerous Goods Regulations* provided requirements for packaging a shipment to classify, mark, pack, label, and document dangerous goods to meet international requirements. Key issues in shipping biological materials include—at a minimum—the following:

- maintaining the sample integrity (especially metabolic viability);
- some identification of the sample if possible (determining appropriate Category A, Category B, or toxin);
- packaging requirements (packaging corresponding to category such as Category A must consist of three components: [1] a primary receptacle[s]; [2] a secondary packaging; and [3] a rigid outer packaging); and
- documentation (International Air Transport Association Shipper's Declaration for Dangerous Goods, DD Form 2890, DoD Multimodal Dangerous Goods Declaration, APHIS/CDC Form 2, Request to Transfer Select Agents and Toxins, and any import or export permits required).

Other considerations for shipping biological samples may exist[5,23,24] and typically require personnel who have been trained and are certified to package hazardous materials for shipment (including but not limited to Transport of Biomedical Materials at https://phc.amedd.army.mil/Pages/CourseDetails.aspx?CourseID=89 [valid September 2016]). Specific specimen collection and handling guidelines for the bioterrorism agents are available from CDC and the American Society for Microbiology (see http://emergency.cdc.gov/bioterrorism/ or http://www.asm.org/index.php/guidelines/sentinel-guidelines; both valid September 2016).

Culture-Based Microbiological Methods

Microbes that cause infectious disease are an example of a classic host–parasite relationship. Suspecting, or even having some evidence of a microbe's ability to produce disease, is still inferential science. Having

TABLE 26-4

INTERNATIONAL AND DOMESTIC STANDARDS FOR SHIPPING

International	Domestic
United Nations Economic Commission for Europe "Recommendations on the Transport of Dangerous Goods," also called the "Orange Book" 2009 (http://www.unece.org/trans/danger/publi/unrec/rev16/16files_e.html; valid February 2014)	49 CFR transportation (highway transportation regulations)
European Agreement concerning the International Carriage of Dangerous Goods by Road (ADR) (http://www.unece.org/trans/danger/publi/adr/adr_e.html; valid February 2014)	42, 7, & 9 CFR for select agents
Final Governing Standards (each country; DoD Instruction 4715.5, Management of Environmental Compliance at Overseas Installations) (https://www.fedcenter.gov/Login/index.cfm?pge_id=3739&NotAuthorized=1&returnto=%2Fprograms%2Fcompliance%2Ffgs%2Findex%2Ecfm%3F; valid February 2014)	9 and 21 CFR for biological products
International Air Transport Association Dangerous Goods Regulations (2014; 55th edition) (http://www.iata.org/publications/dgr/Pages/manuals.aspx; valid February 2014)	US Postal Service Domestic Mail Manual (10.17 Infectious Substances, Hazard Class 6, Division 6.2) and International Mail Manual (135.1 Infectious Substances)
International Civil Aviation Organization Regulations (http://www.icao.int/safety/DangerousGoods/Pages/default.aspx; valid February 2014)	DoD 4500.9-R, Defense Transportation Regulation; Part II, Chapter 204
International Maritime *Dangerous Goods Code* (http://www.imo.org/blast/mainframe.asp?topic_id=158; valid February 2014) (for maritime shipments)	Air Force Manual 24-204/(Interservice) TM 38-250 NAVSUP PUB/505 MCO P4030.19J DLAI 4145.3 (2012), *Transportation; Preparing Hazardous Materials for Military Air Shipments*
World Health Organization Guidelines Guidance on Regulations for the Transport of Infectious Substances 2007–2008 (http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_EPR_2007_2cc.pdf valid February 2014) *Laboratory Biosafety Manual* - Third Edition (http://www.who.int/csr/resources/publications/biosafety/WHO_CDS_CSR_LYO_2004_11/en/; valid February 2014)	Army Regulation 50-1, *Nuclear and Chemical Weapons and Materiel Biological Surety* (2008)
Globally Harmonized System of Classification and Labeling of Chemicals (https://www.osha.gov/dsg/hazcom/global.html; valid July 2016)	Army Regulation 190-17, *Biological Select Agents and Toxins Security Program* (2009)

CFR: Code of Federal Regulations; DLAI: Defense Logistics Agency Information; DoD: Department of Defense; MCO: Marine Corps Publications; NAVSUP PUB: Navy Supplement Publication; TM: Technical Manual

unequivocal proof of a specific etiological agent as the cause of an infectious disease requires the application of conventional microbial culture to validate Koch's postulates (the four standards of a logical chain of experimental evidence designed to establish a causal relationship between a causative microbe and a disease). Microorganisms can cause tissue damage (disease) by releasing a variety of toxins or destructive enzymes into the host. Although a number of ways exist to obtain indirect evidence of a microbe's effect on the host, propagating the causative microbial agent is still considered the gold standard for linking a specific microbial agent to the disease status.

Specific guidelines for identifying bioterrorism agents can be obtained from the CDC (http:\www.bt.cdc.gov) or the American Society for Microbiology (http://www.asm.org/index.php/guidelines/sentinel-guidelines). Guidelines for identification of additional agents that cause other infectious diseases can be found in diagnostic microbiology textbooks. Although the ability to propagate infectious disease microbes in routine culture has been available for more than a century,

many bioterrorism and infectious disease agents—especially the viruses—are not always easily cultured. In addition, culturing a specific microbial agent from a clinical sample is often routine; culturing the same microbial agent from an environmental sample is manyfold more difficult. In either case, knowing which microbial agent(s) is needed will greatly help to create the right conditions for propagation. A physician's clinical observations or medical intelligence should help guide the analytical plan (see Table 26-3).[18,25]

The bioterrorism and infectious disease agents are separated into aerobic and anaerobic bacterial agents and viruses. Fungal and parasitic microbial agents are not often encountered as bioterrorism and infectious disease agents targeted against humans. Most aerobic bacterial threat agents can be isolated by using three common clinical bacteriological media: (1) 5% sheep blood agar (SBA); (2) MacConkey agar; and (3) chocolate agar (CHOC). Cystine heart agar supplemented with 5% sheep blood has been suggested as a preferred medium for *F tularensis,* but CHOC agar usually suffices in clinical samples. Although *Brucella* agar was developed as a preferred medium for *Brucella*, improvements in SBA and CHOC agars support the growth of fastidious microorganisms such as *Brucella.* Nonselective SBA supports the growth of most bacterial agents, including *B anthracis*, *Brucella*, *Burkholderia*, and *Y pestis*. MacConkey agar, which is the preferred selective medium for gram-negative *Enterobacteriaceae*, supports *Burkholderia* and *Y pestis*. Liquid medium, such as trypticase soy broth, can also be used followed by subculturing to SBA or CHOC when solid medium initially fails to produce growth.

Anaerobic organisms (those organisms that do not require oxygen for growth; some of which may react negatively or even die if oxygen is present), such as *Clostridium* species, require the use of anaerobic media and methods. Anaerobic methods reduce the exposure of microorganisms to molecular oxygen through the use of anaerobic jars or anaerobic chambers, and use culture media that are especially designed to dissolve or deplete oxygen, allowing the anaerobes to propagate. The liquid medium thioglycollate readily supports anaerobic microorganisms and should be considered a routine medium if *Clostridium* species could be encountered.

The use of multiple bacteriological media is recommended both for redundancy as well as an aid to initial notification. Propagation of viruses is more complex and usually takes longer than those for bacteria. Since viruses are obligate intracellular parasites, propagation in various host systems is required. Most readily viruses are typically propagated in cultures of various cell lines, but laboratory animals and embryonated eggs are also used. Although no single cell culture is sensitive to all the viruses encountered, Vero (African green monkey kidney) cells are commonly used for many of the viruses (Table 26-5).

Cells used for propagating viruses require growth at an appropriate temperature and gas mixture (typically, 37°C, 5% CO_2 for mammalian cells) in an incubator. In addition, cell cultures also require special growth media that have stringent requirements for pH, glucose, antibiotics, growth factors, and other nutrients. Growth factors used to supplement media are often derived from the serum of animal blood, such as fetal bovine serum. Cell plating density (number of cells per volume of culture medium) and inoculation density of the virus are critical factors. Viruses manifest their presence in cell culture by different mechanisms including cellular degeneration (cytopathic effect), plaque formation, and metabolic inhibition testing. Some viruses require other means to demonstrate their presence in cell culture including fluorescent antibody testing or nucleic acid amplification methods.

Automated Identification Systems

Many automated identification systems are commercially available that have some capability to identify the major bacterial biological threat agents (*B anthracis*, *Brucella* spp, *Burkholderia mallei*, *Burkholderia pseudomallei*, *F tularensis*, and *Y pestis*). These systems include the BioMérieux (Durham, NC) VITEK 2, Siemens (Tarrytown, NY) MicroScan, MIDI Sherlock Microbial Identification System (Newark, DE), Trek (Cleveland, OH) ARIS 2X, Biolog (Hayward, CA), and the Bruker (Billerica, MA) Biotyper matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). The Becton Dickinson (Franklin Lakes, NJ) Phoenix Automated Microbiology System does not appear to be capable of identification of the major bacterial biological threat agents listed. An advantage to the automated identification systems is that if a laboratory is routinely using one of these commercial systems, personnel are already trained and reagents are typically on-hand. The primary disadvantage is that often false-positives or false-negatives occur, including misidentifications as another organism (Table 26-6). Although some identifications on some systems are problematic, identification of some agents by the automated systems are very accurate and often highly discriminatory. The identification of *B anthracis* and *F tularensis* by the MIDI Sherlock Microbial Identification System is very specific and an accepted method.[26–28] Blind acceptance of results from one of the automated commercial systems, however, needs to be avoided, and results need to be substantiated, or refuted, by other assay information.

TABLE 26-5

VIRAL HEMORRHAGIC FEVER CULTURE INFORMATION

	Viral Hemorrhagic Fever				
	Virus	**Endemic Area**	**Mortality**	**Cells and Incubation Time**	**Growth Characteristics**
Arenaviruses	Lassa virus	West Africa	1%–2%	Vero E6–Vero: 3–5 d	No CPE; requires 2nd assay; plaques
	Junin	Argentinian pampas	30%	Vero: 3–5 d	No CPE; requires 2nd assay; plaques are difficult, but possible
	Machupo	Bolivia	25%–35%	Vero E6: 3–5 d	No CPE; plaques fine
Bunyaviruses	Crimean-Congo hemorrhagic fever virus	Africa, SE Europe, Central	30% <0.5%	SM 3–14 d; possible to passage in E6, SW13, or CER cells after initial isolation, but may require >1 blind passages	Plaque assays just as difficult
	Rift Valley fever	Asia, India, Africa		Vero: 2–4 d	CPE/plaques
	Hanta virus (Hantaan, Dobrava, Seoul, Puumala, Sin Nombre Andes)	Europe, Asia, South America (rare)	5% for HFRS	Vero E6: 10–14 d	No CPE; requires 2nd assay such as IFA or PCR; often requires blind serial passages to isolate; hard to plaque
Filoviruses	Ebola virus	Africa, Philippines (Ebola Reston)	50%–90% for Sudan/ Zaire	Vero E6: 6–12 d	CPE/plaques
	Marburg virus	Africa	23%–70%	Vero E6: 6–12 d	CPE/plaques
Flavivirus	Yellow fever virus	Africa, South America	Overall 3% to 12%, 20% to 50% if severe second phase develops	MK2 cells (also BHK21): 3–6 d	Little to no CPE; requires 2nd assay such as PCR or IFA to confirm; plaques fine in Vero cells
	Kyasanur Forest disease virus	Southern India	3%–5%	Vero/Vero E6 SM: 3–6 d	CPE/plaques
	Omsk hemorrhagic fever virus	Siberia	0.2%–3%	Vero/Vero E6 SM: 3–6 d	CPE/plaques

CER: chicken embryo related; CPE cytopathic effect; HFRS: hemorrhagic fever with renal syndrome; IFA: immunofluorescence assay; PCR: polymerase chain reaction; SE: southeast; SM: suckling pig

Although not an automated identification system, identification of bacteria with sequence data of rRNA genes (16S or 23S) needs to be mentioned. Carl Woese pioneered this use of 16S rRNA in the late 1970s for use in phylogenetic studies.[29] 16S rRNA gene sequencing has become a standard reference method for identification of many microbes. Bacterial 16S rRNA gene sequences are available on public databases such as the National Center for Biotechnology Information and the Michigan State University Ribosomal Database Project. Commercially, Applied Biosystems (Foster City, CA) sells 16S rDNA bacterial identification kits under the MicroSeq name that provide standardized reagents and protocols, but they are not yet FDA approved for direct patient care. Although implementation in a routine clinical microbiology laboratory has several drawbacks for microbial identification (time and cost predominately), the accuracy and practicality for many

TABLE 26-6

AUTOMATED IDENTIFICATION SYSTEMS FOR BIOLOGICAL THREAT AGENTS

		Siemens MicroScan		MIDI Sherlock Microbial Identification System	Trek ARIS 2X	Biolog	Bruker Biotyper*
	bioMérieux/ VITEK 2	Rapid Neg ID/Type 3 Plate	Neg ID Type 2 Plate	Biodefense Library 3.0/BTR3 and RBTR3 Instant FAME	GNID Plate	Dangerous Pathogen Identification Database†	Security-Relevant Library
Bacillus anthracis	Yes – BCL card	—	—	Yes‡	—	Yes – GP plate	Yes
Brucella spp	Yes – GN card§		Yes	Yes	Yes	Yes – GN plate	Yes
Burkholderia mallei/ pseudomallei	Yes – GN card¥	—	—	Yes	—	Yes – GN plate	Yes¶
Francisella tularensis	Yes – GN card§, ¥	—	—	Yes	Yes	Yes – GN plate	Yes
Yersinia pestis	Yes – GN card	Yes	Yes¥	Yes	Yes	Yes – GN plate	Yes*

*Another system, bioMérieux VITEK MS is similar.
†GENIII plate has been evaluated for all biological threat agents, but database is not commercially available.
‡AOAC INTERNATIONAL cleared for *Bacillus anthracis* ID.
§Noted as a species that may be nonreactive.
¥Known false results for this organism on this system.
¶Differentiation of *Burkholderia mallei* and *Burkholderia pseudomallei* may not be possible.
AOAC: Association of Official Analytical Chemists
BCL: *Bacillus* identification card
GN: Gram negative
GNID: Gram-negative ID
GP: Gram positive
ID: identification
Neg: negative
FAME: fatty acid methyl esterification

of the biological threat agents is useful. But like all systems, there are limitations to full implementation, predominately in that *B anthracis*, *Brucella* species, and *Y pestis* are often unable to be differentiated from near neighbors with sufficient resolution to make the system practical.

Antibiotic and Antimicrobial Susceptibility Testing

A principal reason for propagation of bioterrorism or infectious disease agents in culture is to screen the agent for antibiotic or antimicrobial agent resistance or susceptibility. Although most of the bacterial biological threat agents have well-characterized susceptibility to antibiotics (Table 26-7), it will be critical to distinguish those organisms that acquire natural or laboratory modifications to normal or traditional antimicrobial susceptibility.[30] Strains of *B anthracis*,[31–33] *Brucella abortus*, *Burkholderia* spp,[30] *F tularensis*,[34,35] and *Y pestis*[36,37] have been reported to have natural antimicrobial drug resistance, including multiple drug resistances.[38–43] The Clinical and Laboratory Standards Institute (www.clsi.org) has published standard protocols that include the biological threat agents to ensure accuracy and reproducibility of results. For the biological threat agents, classical minimum inhibitory concentration determinations are the preferred method.[44] Although commercial antibiotic susceptibility testing devices are available,[45,46] they have not been standardized to ensure correspondence to the reference method. The CDC LRN does include the use of the Epsilometer test (E-test) for antimicrobial susceptibility testing of selected microorganisms. The E-test is a direct quantification agar dilution method[47] that has been adopted by many laboratories because of its ease of use and quantification capabilities. Molecular methods that screen for unique genetic markers of resistance have been developed[37,48–54]; however, molecular analysis approaches can be cumbersome when multiple loci are involved[50,51] and do not always correlate with therapeutic effectiveness nor laboratory data.[30] DNA microarrays offer the potential for simultaneous testing for specific antibiotic resistance genes, loci, and markers,[49,50,55] but are not sufficiently developed for routine use.

Microbial Culture Versus Rapid Methods

With the introduction of newer rapid methods for biological threat agent detection and the codification of the term biomarkers in the military doctrine, there has been avoidance on the discussion of classical microbiological culture in the detection of biological threat agents. Classical microbiology culture, whether for bacteria or viruses, has been stigmatized as archaic and overly time consuming. The concept of obtaining a result in less than an hour—and being able to do something with that result—has taken center stage. Although the newer rapid methods for biological threat agent detection have matured over the past decade, there are still problematic areas in the sole reliance on these newer methods.

Current concepts of operations for theater validation laboratories are for multiple technologies that do not necessarily include culture of the organism. Most often, the use of nucleic acid amplification (through PCR) and immunoassays are the predominant methods for rapid identification. Operation of a theater validation laboratory with PCR and immunoassay technologies does not require the containment of a BSL-3 facility.

To cause disease, microbial agents must be living or toxin agents must be biologically (metabolically) active. Unless an identification of a biological agent is based on some metabolic method, in the absence of morbidity or mortality, there is a possibility that the implicated agent has been inactivated.[16] Inactivation of biological materials, especially in nonclinical

TABLE 26-7

STANDARD ANTIBIOTIC SUSCEPTIBILITY TESTING FOR BIOLOGICAL THREAT AGENTS

Bacillus anthracis	*Brucella* spp	*Burkholderia mallei/pseudomallei*	*Francisella tularensis*	*Yersinia pestis*
Penicillin	Gentamicin	Doxycycline	Gentamicin	Gentamicin
Doxycycline	Streptomycin	Tetracycline	Streptomycin	Streptomycin
Tetracycline	Doxycycline	Imipenem	Doxycycline	Doxycycline
Ciprofloxacin	Tetracycline	Amoxicillin-clavulanate	Tetracycline	Tetracycline
	Trimethoprim/ sulfamethoxazole	Trimethoprim/ sulfamethoxazole	Ciprofloxacin	Ciprofloxacin
			Levofloxacin	Chloramphenicol
			Chloramphenicol	Trimethoprim/ sulfamethoxazole

samples, readily occurs and culture (or multimetabolic assays for toxins) is the only way to ensure that an implicated biological agent is actually capable of causing disease.

CDC LRN reference laboratories typically include the use of BSL-3 facilities because the CDC LRN identification requires identification based on culture of the organism(s). Current DoD doctrine, at the theater validation level, does not include culture as a requirement. The CDC LRN, however, does not include viral diagnostics/detection capabilities other than the inclusion of smallpox and other orthopoxviruses. In some areas of operations, consideration for viral threat agents is just as high, if not higher, than for the more traditional bacterial agents. Although deployed assets for the diagnostic/detection of viruses are not robust for practical reasons, consideration for those agents must be included in operations planning. Bacterial culturing can be done in BSL-2 facilities for the majority of the biological threat agents (Department of the Army Pamphlet [DA PAM] 385-69)[56]; however, it invokes enhanced requirements on facilities engaged in culturing any organisms, even those less than BSL-3. Any laboratory doing culture work will have to comply with all the provisions of that reference. Laboratories not doing culture work do not invoke the requirements of DA PAM 385-69.

Another consideration for inclusion of microbial culturing technologies includes the ability to provide sufficient samples for forensic science analysis and attribution. Without the propagation of the causative agents, the ability to conclusively confirm the agent as well as the ability to share samples among attribution laboratories will be greatly hindered.

Integration of In Vivo and In Vitro Diagnostic Tests

Integrated diagnostics, or orthogonal testing, is a recommended testing strategy for both clinical as well as environmental samples. Orthogonal diagnostic testing is the key to improving the reliability of rapid diagnostic technologies. Orthogonal testing refers to tests that are statistically independent or nonoverlapping but—in combination—provide a higher degree of certainty of the final result. Although orthogonal testing is not a standard perspective in the clinical diagnostic industry, the concept and its application are paramount when investigating some infectious agents. Any single detection technology has a set of limits with regard to sensitivity and, most importantly, specificity. Orthogonal testing seeks to overcome the inherent limitations of individual test results with the strength of data combinations.[18] The application of orthogonal diagnostic testing uses an integrated testing strategy where more than one technology, technique, or biomarker is used to produce diagnostic results, which are then interpreted collectively (Figure 26-1).

Immunodiagnostic Methods

An integrated approach to agent detection and identification, using both immunological and nucleic acid-detection, will provide the most reliable laboratory data and is essential for a complete and accurate disease diagnosis.[18] Understanding the strengths and weaknesses of each assay is paramount in the interpretation of results. Nucleic acid-detection assays are exquisitely sensitive and specific; this is the strength of the assay, but it can also be a weakness in particular situations. Immunodiagnostic assays are comparatively less sensitive, but have broader specificity; this is a weakness of the assay, but it can also be a strength in certain situations.

In an orthogonal system, the advantages of the nucleic acid and immunological assays will offset the disadvantages. Detection of an endemic pathogen will rely on the high sensitivity of the nucleic acid-detection assay; however, for a newly emerging genetic variant the specificity of the nucleic acid-detection assay may result in a false negative. A detection system that incorporates immunodiagnostic assays will detect the variant with the broader specificity of antibodies. This can be illustrated with the detection of the newest ebolavirus, Bundibugyo. Initially, PCR-based assays failed to detect the virus because of the genetic variation. Only when the less sensitive but more broadly reactive antigen detection and capture immunoglobulin M ELISAs were used was the virus detected and identified as an ebolavirus.[57] Clearly, both immunodiagnostic and nucleic acid-detection assays are vital when detecting pathogens that exhibit genetic variation whether natural or intentionally engineered.

Immunodiagnostic techniques diagnose disease by detection of agent-specific antigens and/or antibodies present in clinical samples. The most significant problem associated with development of an integrated diagnostic system is the inability of immunodiagnostic technologies to detect agents with sensitivities approaching those of more sensitive nucleic acid-detection technologies. These differences in assay sensitivity increase the probability of obtaining disparate results, and they could therefore actually complicate medical decisions. However, continued advances in immunodiagnostic technologies provide the basis for developing antigen- and antibody-detection platforms capable of meeting requirements for sensitivity, specificity, assay speed, robustness, and simplicity. Detection of specific proteins or other antigens or host-produced antibodies

directed against such antigens constitutes one of the most widely used and successful methods for identifying biological agents and for diagnosing the diseases they cause. Nearly all methods for detecting antigens and antibodies rely on production of complexes made of one or more receptor molecules and the entity being detected (Figure 26-5).

Diagnosing disease using immunodiagnostic technologies is a multistep process involving formation of complexes bound to a solid substrate. This process is like making a sandwich in which detecting the biological agent or antibody depends on incorporation of all of the sandwich components. The assays are relatively simple and robust, but elimination of any one part of the sandwich results in a failure and a negative response. Primary ligands used in most immunoassays are polyclonal or monoclonal antibodies or antibody fragments. Generally, the first step in an immunodiagnostic assay is binding one or more antibodies for the target of interest onto a solid support. Immunoassays are either heterogeneous or homogeneous depending on the nature of the solid substrate. A heterogeneous assay requires physical separation of bound from unbound reactants by using techniques such as washing or centrifugation. These types of assays can remove interfering substances and are, therefore, usually more specific.

Heterogeneous assays require more steps and increased manipulation that cumulatively affect assay precision. A homogeneous assay requires no physical separation but may require pretreatment steps to remove interfering substances. Homogeneous assays are usually faster and more conducive to automation because of their simplicity. However, the cost of these assays is usually greater because of the types of reagents and equipment required.

Once the test sample is reacted with the capture element, the final step in any immunoassay is detection of a signal generated by one or more assay components. This detection step is typically accomplished by using antibodies bound to (or labeled with) inorganic or organic molecules that produce a detectable signal under specific chemical or environmental conditions. The earliest labels used were molecules containing radioactive isotopes. However, radioisotope labels have generally been replaced with less cumbersome labels such as enzymes. Enzymes are effective labels because they catalyze chemical reactions, which can produce a signal. Depending on the nature of the signal, reactants may be detected visually, electronically, chemically, or physically. A single enzyme molecule can catalyze many chemical reactions without being consumed in the reaction; therefore, these labels are effective at amplifying assay signals. Most common

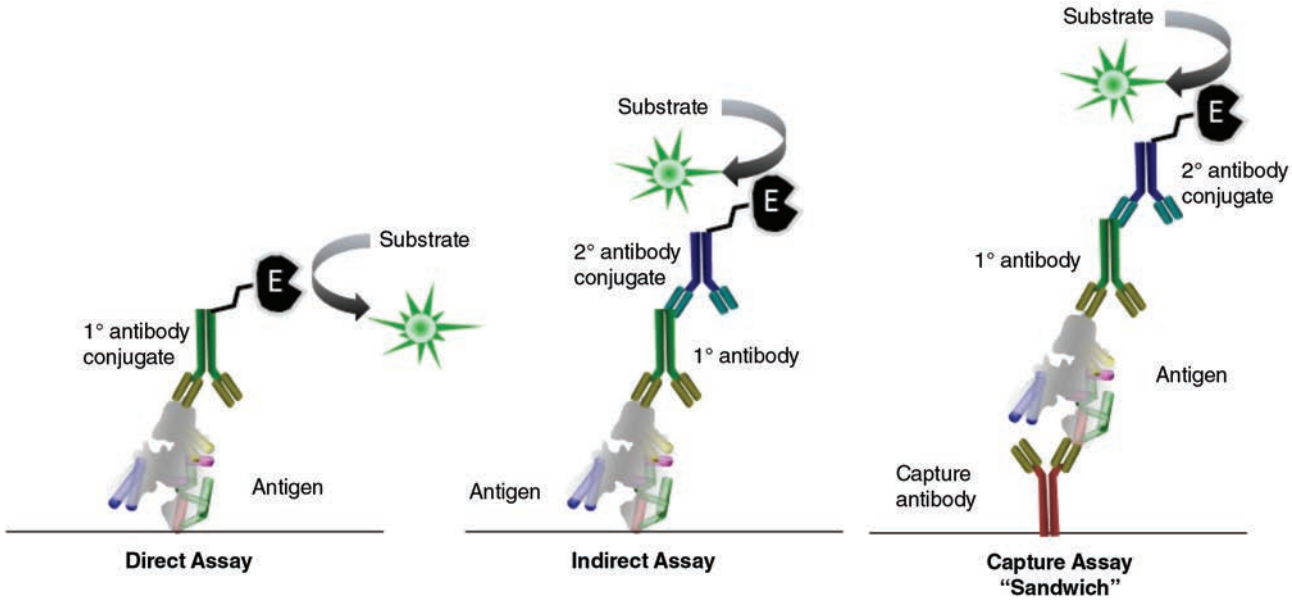


Figure 26-5. Representation of common enzyme-linked immunosorbent assay formats. The assay can be configured to detect antigen or antibodies. The target of interest (direct and indirect assays) or a capture antibody (sandwich assay) is immobilized by direct adsorption to a solid support such as a 96-well plate or magnetic bead. Detection of the target is accomplished using an enzyme-conjugated primary antibody (direct assay) or a matched set of unlabeled primary and conjugated secondary antibodies (indirect and sandwich assays).
E: enzyme; 1°: first degree; 2°: second degree

enzyme-substrate reactions used in immunodiagnostics produce a visual signal that can be detected with the naked eye or by a spectrophotometer.

Fluorescent dyes and other organic and inorganic molecules capable of generating luminescent signals are also commonly used labels in immunoassays. Assays using these molecules are often more sensitive than enzyme immunoassays, but require specialized instrumentation and often suffer from high background contamination resulting from intrinsic fluorescent and luminescent qualities of some proteins and light-scattering effects. Signals in assays using these types of labels are amplified by integrating light signals over time and cyclic generation of photons. Other commonly used labels include gold, latex, and magnetic or paramagnetic particles. Each can be visualized by the naked eye or by instruments and are stable under a variety of environmental conditions. However, these labels are essentially inert and therefore do not produce an amplified signal. Signal amplification is useful and desirable because it results in increased assay sensitivity.

Advances in the fields of biomedical engineering, chemistry, physics, and biology have led to an explosion of new diagnostic platforms and assay systems that offer great promise for improving diagnostic capabilities. An overview of technologies currently being used for identification of biological agents and either being used for diagnosing or being developed for use in diagnosing the diseases they cause will be presented.

Enzyme-Linked Immunosorbent Assay

Since the 1970s, ELISA has remained a core technology for diagnosing disease caused by a wide variety of infectious and noninfectious agents. As a result, ELISA is perhaps the most widely used and best understood immunoassay technology. Assays, which have been developed in many formats, can be designed to detect either antigens associated with the agents themselves or antibodies produced in response to infection. ELISAs that detect biological agents or agent-specific antibodies are heterogeneous assays that capture agent-specific antigen or host-derived antibody onto a plastic multi-well plate by an antibody or antigen previously bound to the plate surface (capture element). Complexed antigen or antibody is then detected using a secondary antibody (detector antibody). The detector antibody can be directly labeled with a signal-generating molecule such as in a direct ELISA, or it can be detected with another antibody that is labeled with an enzyme such as in an indirect or capture (sandwich) ELISA formats. These enzymes catalyze a chemical reaction with substrate that results in a colorimetric change. Intensity of this color can be measured by a modified spectrophotometer that determines the optical density of the reaction using a specific wavelength of light. In many cases, the assay can be interpreted without instrumentation by simply viewing the color that appears in the reaction vessel.

The major advantages of ELISAs are their ability to be configured for a variety of uses and applications. ELISAs can be used in field laboratory settings, but they require power for temperature-controlled incubators and refrigerators and other ancillary equipment needs. In addition, ELISAs:

- are commonly used and understood by clinical laboratories and physicians;
- are amenable to high-throughput laboratory use and automation;
- do not require highly purified antibodies; and
- are relatively inexpensive to perform.

The major disadvantages are that they are labor intensive, temperature dependent, have a narrow antigen concentration dynamic range that makes quantitation difficult, and are relatively slow.

At the US Army Medical Research Institute of Infectious Diseases, antigen-detection ELISAs have been developed for nearly 40 different biological agents, and antibody-detection ELISAs have been developed for nearly 90 different agents. All of these assays were developed to use the same solid phase, buffers and other reagents, with similar incubation periods, incubation temperatures, and general procedures (Table 26-8). Although significant variation exists in assay limits of detection, ELISAs typically are capable of detecting as little as 1 ng of antigen per ml of sample.

Electrochemiluminescence

Immunodiagnostic technologies based on ECL detection are of continued military interest. ECL technology, commercially developed by BioVeris (Gaithersburg, MD), was incorporated into a field ready immunodiagnostic system, the M1M. The assay formats are similar to those of ELISA; however, magnetic beads serve as the solid support and magnets are used to concentrate target agents. The detection of target uses a chemiluminescent label (ruthenium, Ru). The small size of Ru (1,057 kDa) makes it easily conjugated to any protein ligand (antigen or antibody) using standard chemistries without affecting immunoreactivity or solubility of the protein. The heart of the M1M ECL analyzer is an electrochemical

TABLE 26-8

COMPARISON OF IMMUNODIAGNOSTIC METHODS

	ELISA	ECL	Luminex		HHA
Antibody Requirements					
Purity	None	Required	Required		Required
Labeling	None	Biotin/ruthenium	Biotin/beads		Beads
Assay Parameter					
Coating time	12 h	0	0		0
Incubation time	3.5 h	15 m	30 m		15 m
Read time	1 sec/well	1 m/tube	20–120 sec/well		30 sec
No. of steps	5	1	1		1
No. of buffers required	3	1	1		1
Specialized reagents	Conjugate Substrate	Assay buffer Cell cleaner	Sheath fluid		Sample buffer
Solid phase used	Microtiter well	Magnetic bead	Colored latex bead		Nitrocellulose
Reaction	Bound	In solution	In solution		Bound
Detector label used	HRP	Ru	PE		Gold
Detection method	Colorimetric	Chemiluminescence	Fluorescence		Visual
Amount of sample per test	100 ml	50 ml	50 ml		200 ml
Prozone	No	Yes	Yes		No
Sample matrix effects	No	Yes	Yes		Yes
Multiplexing	No	No	Yes		Potential
Intraassay variation (%)	15%–20%	2%–12%	10%–25%		Undetermined
Potential for PCR analysis	Yes	Yes	Yes		Yes
Limit of Detection (per ml)			**Single**	**Multiplexed**	
Y pestis F1 (CFU)	250,000	500	62,500	125,000	ND
Staphylococcal enterotoxin B (ng)	0.63	0.05	3.13	6.25	ND
Venezuelan equine encephalitis virus (PFU)	1.25×10^7	1.0×10^7	3.13×10^8	6.25×10^8	1×10^8

CFU: colony-forming unit; ECL: enhanced chemiluminescence; ELISA: enzyme-linked immunosorbent assay; HHA: hand held assay; HRP: horseradish peroxidase; ND: not detected; PCR: polymerase chain reaction; PE: phycoerythrin; PFU: plaque-forming unit; Ru; ruthenium

flow cell with a photo-detector placed just above the electrode. A magnet positioned just below the electrode captures the magnetic bead-Ru-tagged immune complex and holds it against the electrode. Application of an electric field results in a rapid electron transfer reaction between the substrate (tripropylamine) and the Ru. Excitation with as little as 1.5 V results in light emission, which in turn is detected by a charge-coupled device camera. The system's strengths come from its speed, sensitivity, accuracy, and precision over a wide dynamic range. Magnetic beads provide a greater surface area than conventional surface-binding assays like ELISA. The reaction does not suffer surface steric and diffusion limitations encountered in solid-phase immunoassays; instead it occurs in a turbulent bead suspension, thus allowing for rapid reaction kinetics and short incubation time. Detection limits as low as 200 fmol/L are possible with a linear dynamic range that can span six orders of magnitude.[58,59]

Assay configurations can be identical to ELISA, direct, indirect, or sandwich assays. For antigen detection assays, the beads are coated with capture antibody, whereas for antibody detection assays the beads are coated with antigen or capture antibody. The coated paramagnetic beads, in the presence of biological agent (target), form immune complexes that are detected by the Ru-conjugated detector antibody. After a short 15-minute incubation period the analyzer draws the sample into the flow cell, captures and washes the magnetic beads, and measures the electrochemiluminescent signal (up to 1 minute per sample cleaning and reading time). Conveniently, the reagents can be lyophilized. The system uses 96-well plates that allow high sample throughput.

The ECL system effectively can detect staphylococcal enterotoxin B, ricin toxin, botulinum toxin, *F tularensis*, *Y pestis* F1 antigen, *B anthracis* protective

antigen (PA) and capsule, and Venezuelan equine encephalitis virus.[18,60–63] The system, which had been demonstrated in field settings, was used as one part of an integrated diagnostic system in several deployable and/or deployed laboratories. In 2007, Roche (Basel, Switzerland) acquired BioVeris to expand its ECL-based Elecsys Systems, which ultimately led to the demise of the M1M platform and its use by the DoD. The platform remains in use, but Roche is no longer producing reagents and the system will be forced into obsolescence when supplies are no longer available. Critical assay performance characteristics and detection limits from three typical ECL agent-detection assays are shown in Table 26-8.

Meso Scale Diagnostics (Rockville, MD) has developed a line of immunodiagnostic instruments based on the ECL technology. Unlike the M1M that was single plex, analyzing a single sample for a single target, the MSD instrument is capable of multiplex analysis, analyzing for multiple targets on a single sample. The Meso Scale Diagnostics MULTI-ARRAY technology uses ECL to detect binding events on patterned arrays. In multiwell microplates, capture antibodies are bound to carbon electrodes integrated into the bottom of the plate. The plates can have up to 10 electrodes per well, with each electrode coated with a different capture antibody. Similar to the sandwich ELISA, the target of interest is captured on the electrode and detected by the target-specific Ru-conjugated detector antibody. As in the M1M system, electrochemical stimulation results in the Ru label emitting light at the surface of the electrodes, from which the concentration of target associated with the particular electrode can be determined.

Evaluation of the technology at USAMRIID found sample testing in simple matrices, like the high volume air handler buffer, worked well, but the assays suffered from increased backgrounds in more complex matrices, like blood or serum. The ECL analyzer PR2 is available in a manual configuration, Model 1800, and a fully automated configuration, Model 1900, each of which is capable of high-throughput analysis. For environmental testing, the Model 1500 is designed for automated aerosol sample testing.

This multiplexed immunoassay platform has more than 400 assays commercially available for use in clinical, environmental, and research applications, with kits that are specifically designed for biodefense. MSD assays can be customized; however, antibody printing onto the electrodes must be done by the company, rendering laboratory derived tests less flexible, more complicated, and most likely more expensive. The NGDS acquisition program has identified the MSD PR2 instruments for possible inclusion as the immunodiagnostic component in its portable human diagnostic system. Dependence on any single company for both instrument and assays increases the risk to the DoD diagnostic and detection programs, which is reminiscent of the BioVeris experience.[60]

Flow Cytometry

Flow cytometry, the measurement of physical and chemical characteristics of small particles, has many current applications in research and healthcare and is commonplace in most large clinical laboratories. Applications include cytokine detection, cell differentiation, chromosome analysis, cell sorting and typing, bacterial counting, hematology, DNA content, and drug discovery. The technique works by placing biological samples (ie, cells or other particles) into a liquid suspension. A fluorescent dye, the choice of which is based on its ability to bind to the particles of interest, is added to the solution. The suspension is made to flow in a stream past a laser beam. Light is scattered, and the distribution and intensity of scattered light is characteristic of the sample passing through. The wavelength of light is selected such that it causes the dye—bound to the particle of interest—to fluoresce. A computer counts and/or analyzes the fluorescent sample as it passes through the laser beam. Using the same excitation source, fluorescence may be split into different color components so that several different fluorophores can be measured simultaneously and signals interpreted by specialized software. Multiplexed flow cytometry assays have been demonstrated for a variety of cytokine targets.[64] Particles can also be sorted from the stream and diverted into separate containers by applying a charge to the particles of interest.

The Luminex xMAP technology (Austin, TX) has resulted in significant improvements in multiplex flow cytometry-based diagnostics. The xMAP technology is based on polystyrene bead sets encoded with different intensities of red and infrared dyes (unique address to a bead set) and coated with a specific-capture antibody against one of the analytes of interest. Interrogation of the beads by two lasers identifies the spectral property of the bead (address) and hence the associated analyte, in addition to the phycoerythrin labeled secondary antibody against the specific analyte.

The Luminex 100/200 (Austin, TX) and the FLEXMAP 3D systems are flow cytometry-based instruments that can rapidly perform up to 100 tests simultaneously on a single sample. They incorporate three familiar technologies: (1) bioassays, (2) microspheres, and (3) fluorescence. Assays occur in solution; thus, reaction kinetics are rapid and incubation times are shorter. Capture antibodies or ligands are bound to

microspheres labeled with two spectrally distinct fluorochromes. By adjusting the ratio of each fluorochrome, microspheres can be distinguished based on their spectral address. Bioassays are conducted on the surfaces of these microspheres. Detector antibodies are labeled with any of a number of different green fluorescent dyes. This detector-bound fluorochrome measures the extent of interaction that occurs at the microsphere surface; that is, it detects antigen in a typical antigen-detection assay. The instruments use two lasers: one for detection of the microsphere itself, and the other for the detector. Microspheres are analyzed individually as they pass by two separate laser beams, are classified based on their spectral address, and are measured in real time. Thousands (20,000) of microspheres are processed per second resulting in an assay system theoretically capable of analyzing up to 100 different reactions on a single sample in just seconds.

The manufacturer reports assay sensitivities in the femtomole level, dynamic range of 3 to 4 orders of magnitude, and claims results are highly consistent and reproducible.[65] Because the intensity of the fluorescent label is read only at the surface of each microsphere, any unbound reporter molecules remaining in solution do not affect the assay, making homogeneous assay formats possible. The system, which can use tubes as well as 96- and 384-well plates, can be automated. In addition to the Luminex instrument, a plate shaker and liquid handling devices are required to complete assays. As with most technologies, many different formats can be used. Many multiplexed assay kits are commercially available from different manufacturers for various cytokines, phosphoproteins, and hormones.

The FLEXMAP 3D instrument is capable of high throughput and can be automated, which makes it better suited for a large clinical laboratory. No field-ready versions of the Luminex 100/200 are available, which limits the practical use of this instrument in deployment situations. No commercial or DoD sources for biological threat agent assays are available for this platform.

MAGPIX

Flow cytometry-based systems can be accommodated in large diagnostic laboratories where environmental conditions are controlled and qualified technicians perform preventative maintenance to ensure the flow cells and lasers are clean, aligned, and functioning properly. Recently, the MAGPIX instrument based on the Luminex xMAP technology was introduced. The instrument, which eliminates some of the shortcomings of the flow cytometry-based instruments, has tremendous potential for forward laboratory applications in such resource-limited environments. MAGPIX uses magnetic color-coded microspheres to perform multiplexed assays. Fifty different individually addressable bead sets can be used on an instrument. Instead of interrogating individual microspheres sequentially through flow cytometry, MAGPIX uses magnetic force to move the microspheres to a stage and then images all the magnetic microspheres from that sample at once using a charge-coupled device camera. Three images, each taken with a different filter, are used to discriminate bead sets and determine assay signals. Two images are used to identify the unique bead address and the third image measures the presence of tracer fluorophore, indicating the presence of target analyte. The MAGPIX carries sufficient drive fluid onboard (650 mL) to analyze eight full microtiter plates (768 samples) and has a throughput rate of approximately 96 samples per hour, or 1.6 samples per minute. The system is fully compatible with all magnetic bead-based assays currently performed on the Luminex flow cytometers; all assay, sample, and reagent preparation protocols for both systems are analogous. The sensitivity of the MAGPIX system is similar or identical to the Luminex 100/200 instrument, which can detect ricin in the pg/mL range.

Sensitivities of bead-based assays are typically in the same range as—or in some cases superior to—those obtained in ELISAs.[66,67] Previous limitations in fieldability for the Luminex flow cytometric instruments (large size, susceptibility of the laser alignment to shock or vibration) have also been largely overcome in the new MAGPIX instrument; this latter platform is smaller and more rugged. Per instrument cost has also been significantly decreased, which may also make it more affordable for widespread deployment in forward facilities. Featuring a flexible, open-architecture design, xMAP technology can be configured to perform a wide variety of bioassays quickly, cost effectively, and accurately. Six assays are commercially available for biodefense toxin targets: botulinum toxins A, B, E, F, staphylococcal enterotoxin B (SEB), and ricin.

Hand Held Assays

HHAs are immunodiagnostic assays that are ideally suited for field-based diagnostics. Commonly found on the commercial market, they are simple enough to use and interpret that some types are even approved for over-the-counter use by the FDA; the best known one is the home pregnancy test. HHAs are typically designed on natural or synthetic membranes contained within a plastic or cardboard housing. A capture antibody (for antigen detection) or antigen (for antibody detection) is bound to the membrane and a second

antibody labeled with some visible marker element is placed on a sample application pad. As sample flows across the membrane, antigen or antibody present in the sample binds to labeled antibody and is captured as the complex passes the bound antibody or antigen (Figure 26-6). Colloidal gold, carbon, paramagnetic, or colored latex beads are commonly used particles that create a visible line in the capture zone of the assay membrane.

HHAs are advantageous because they are relatively inexpensive, simple, and require little training to use, and results can be obtained in only 5 to 15 minutes. One of the greatest advantages of HHAs is the lack of reliance on instrumentation and logistical needs associated with those instruments. However, this lack of instrumentation decreases the utility of the tests because results cannot be quantified. To respond to this deficiency, several technologies are available to make these assays more quantitative and have the added benefit of increasing their sensitivity. One technology, produced by Response Biomedical Corporation (Vancouver, British Columbia, Canada), allows for quantitative interpretation of the HHA.[68–71] The Rapid Analyte Measurement Platform (RAMP) cartridges for biodefense can detect *B anthracis*, ricin, botulinum toxin, and smallpox virus. Another method for quantitative detection of antibody/antigen complex formation in HHAs is use of up-converting phosphors.[72,73] Paramagnetic particles have similarly been used in assays: instruments capable of detecting changes in magnetic flux within the capture zone (Quantum Design, San Diego, CA) have proven useful by improving sensitivity by as much as several orders of magnitude over more traditional HHAs.

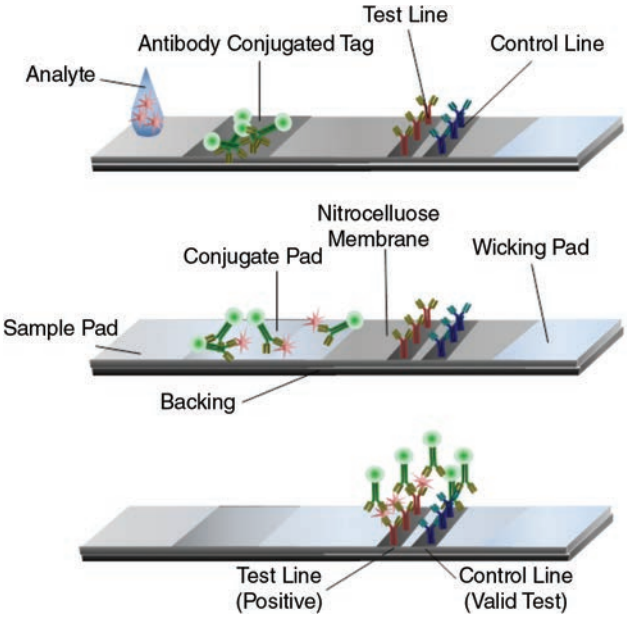


Figure 26-6. Illustration of a typical hand held immunoassay. Photograph: Courtesy of US National Aeronautics and Space Administration.

DoD commonly uses HHAs to detect biological threat agents. The DoD Medical Countermeasure Systems, Critical Reagent Program, a repository for DoD diagnostic reagents, offers lateral flow assays for this purpose. In addition, several commercial companies have begun to market a variety of threat agent tests for use by first responders. However, independent evaluation of these assays has not typically been performed, so data acquired from the use of these assays must be interpreted carefully. Another common disadvantage of HHAs is their inability to incorporate the capability to run a full spectrum of control assays on a single strip assay. Recently, FDA approved two lateral flow assays for the detection of *B anthracis* for use in clinical settings.[74] As with any diagnostic test, understanding its strengths and weaknesses will aid in proper interpretation of the results. HHAs are useful in initial screening of samples for biological threat agents, but results should be followed with confirmatory testing using an orthogonal system.

Future Perspectives

Traditionally, assays for detecting proteins and other nonnucleic acid targets, including antigens, antibodies, carbohydrates, and other organic molecules were conducted using antibodies produced in appropriate host animals. As a result, these assays were generically referred to as immunodiagnostic or immunodetection methods. In reality, numerous nonantibody molecules, including aptamers, peptides, and engineered antibody fragments, are now being used in affinity-based detection technologies.[75–83]

Since an immunodiagnostic assay is directly related to the characteristics of the antibody components used, improved antibodies or antibody-like elements have the potential to significantly improve the sensitivity, specificity, and robustness of the assays. Naturally occurring single domain antibodies (sdAbs) derived from camelids and sharks possess unique properties that could improve present day immunodiagnostics. Through convergent evolutionary processes, both camelid and shark immune systems naturally possess nonconventional antibody subsets composed only of heavy chain homodimers and a single variable domain.[84,85] The variable (V) domains of these antibodies represent the smallest naturally occurring antigen binding domains known. These extremely small (12–15 kDa) sdAbs can target enzyme clefts and

cryptic antigens that conventional antibodies cannot. Unique structural characteristics provide them a high temperature (>60–90°C), proteolytic and pH stability,[86–93] high solubility,[94] and efficient and economical expression in a variety of microorganisms (including *Escherichia coli*).[95] The unique features of these naturally occurring molecules could vastly improve the utility of any immunodiagnostic assay.

Antibody-based biosensors provide the most reliable detection capability across the broadest range of biowarfare agents. They are, therefore, the preferred platform for DoD biosensor applications. However, the fragility of the antibody molecule together with the short shelf life (typically 2 weeks or less) of antibody-based biosensors severely complicates their use outside of a clinical laboratory environment. In addition, the variability in affinity across various antibody systems has precluded the development of multiplexing antibody arrays for biosensor applications. The Defense Advanced Research Projects Agency sponsored the Antibody Technology Program to develop and demonstrate approaches for achieving revolutionary improvements in the stability of antibodies while simultaneously demonstrating the ability to control antibody affinity for use in immunological detection.[96–98] Each performer was supplied with the same starting material, single chain fragments (scFvs), and was asked to improve the antibodies by engineering them for improved stability and affinity. The desired metrics for improvements were decreasing the affinity of the antibody by at least 100-fold and increasing the stability of the supplied antibody such that it maintained its activity at 70°C for 1 hour.

Initially, the performers achieved these requirements in separate proteins before attempting to meet both requirements in one protein. Each group

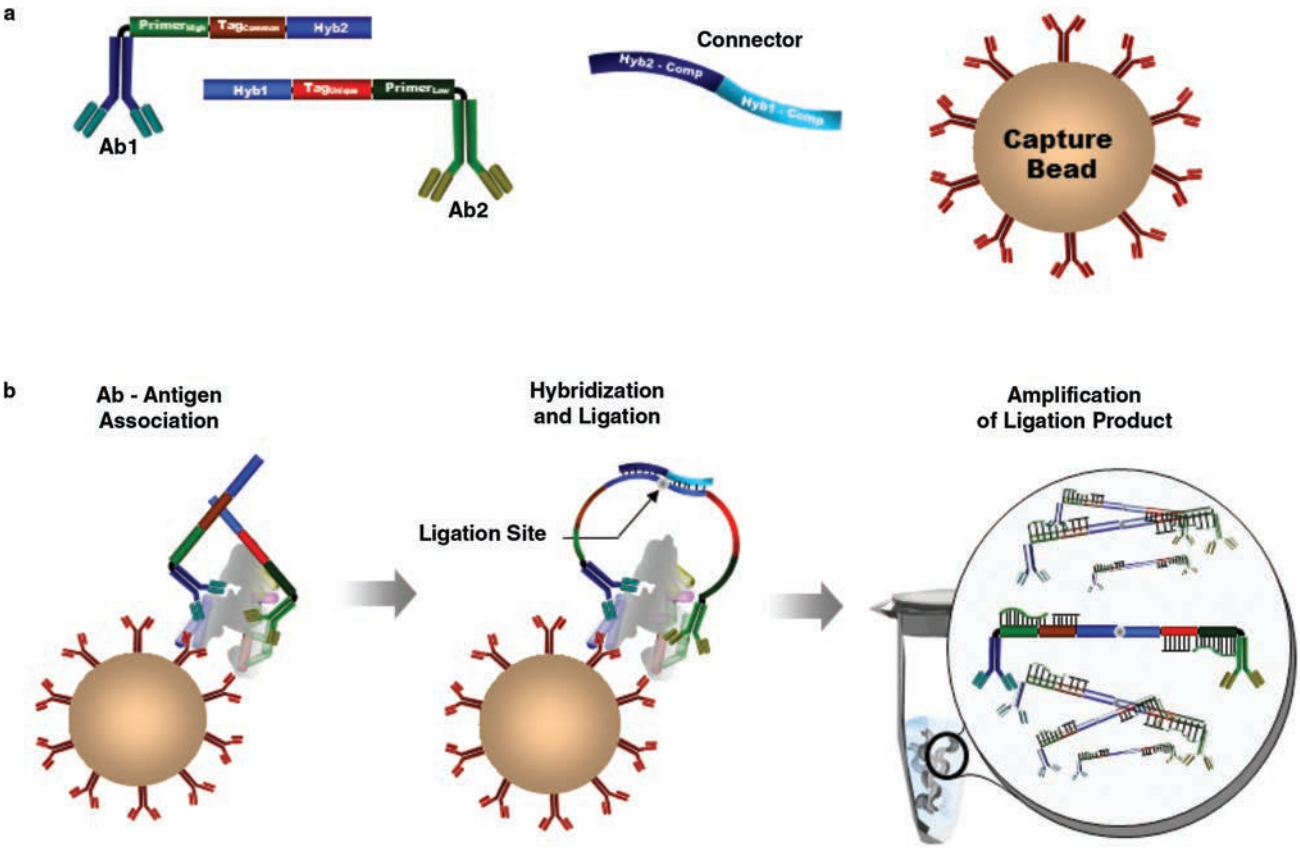


Figure 26-7. Generic overview of PLA reactants and assay. (**a**) In addition to PCR reagents, PLA consists of antibodies to two different epitopes, each labeled with a unique oligonucleotide (proximity probe) and a connector oligonucleotide complementary to the free ends of each proximity probe. Unique to our design is the inclusion of magnetic beads coated with antigen-specific antibodies. (**b**) After formation of a bead/antigen/proximity probe complex, the free 5′ and 3′ ends of the antibody-bound oligonucleotides that are in close proximity to each other hybridize onto the connector oligonucleotide and are covalently joined by DNA ligase. Once joined, these provide a template for PCR amplification. Ab: antibody; Comp: complementary; Hyb: hybridization; PCR: polymerase chain reaction; PLA: proximity ligation assay

approached the solution differently, but produced antibodies with greater binding to the target ligand and improved thermostability. The Antibody Technology Program increased antibody affinity by a factor of 400. Temperature stability of antibody molecules was improved by a factor of 36, which translated into an increased shelf life at room temperature from about 1 month to 3 years. Similarly, antibody survival at 70°C increased from 5 to 10 minutes to 48 hours. By creating these stable antibodies, it was postulated that different variable regions could be grafted onto the developed backbone to increase the stability of antibodies in general, without altering the affinity. These improvements would translate into improved immunodiagnostic assays that would function well in more austere environments, as well as decreasing the cold chain needs for these reagents.

Often the Achilles heel of immunodiagnostic assays is the lower sensitivity when compared to PCR-based assays. Advances in antibody development or engineering can improve antibody characteristics and therefore the resulting assays, but other advancements combine antibody detection with PCR to achieve sensitivity levels equivalent to PCR. Immuno-PCR assays are similar to ELISAs, but substitute the detector antibodies conjugated to enzymes with antibodies that are labeled with DNA.[99] Using label-specific PCR primers, the DNA label is amplified and can result in increased sensitivity of 105-fold. These assays that relied on a single DNA-labeled antibody exhibited high background signals that frequently resulted in false-positive results.[100,101] The proximity ligation assay eliminated the background limitations of immune-PCR by requiring the binding of antibodies to at least two different epitopes on the target antigen.[102] Each antibody is labeled with a specific oligonucleotide containing a PCR primer site and having either a free 5′ or 3′ end (Figure 26-7).

When the antibodies bind the target, the DNA labels are brought into proximity and the two complementary ends hybridize to a connector oligonucleotide with compatible ends. The hybridized strands are joined by DNA ligase and serve as a template for amplification and fluorescent probe detection. The amplified DNA is a surrogate marker for the target protein of interest. The 5′ or 3′ oligonucleotide ends that fail to hybridize completely with connectors cannot be amplified and reduce the background and the possibility of false positives. Proximity ligation assay detection of viruses and bacterium has proven to be more sensitive than ELISA and as sensitive as real-time PCR.[103] In addition, the assays work in a wide variety of biological matrices, serum, plasma, cerebrospinal fluid, cell culture media, and lysates of cells and tissues.[102,104] Improvements in technology and the components of immunodiagnostic assays continue to close the gap in sensitivity between protein detection and nucleic-acid detection making an orthogonal system ever more powerful.

Molecular Detection Methods

PCR is the predominant methodology for detection of molecular signatures. Originally conceived in 1983 by Kary Mullis,[105] the first published application of PCR was by Saiki et al amplifying beta-globin genomic sequences and thus hallmarking the advent of the molecular biology field.[106] In its simplest form, PCR consists of target genomic material, two oligonucleotide primers that flank the target sequence, a heat-stable DNA polymerase, a defined solution of salts, and an equimolar mixture of deoxyribonucleotide triphosphates. This mixture is subjected to repeated cycles of defined temperature changes that facilitate denaturation of the template, annealing of the primers to the target, and extension of the primers so that the target sequence is amplifying. With each cycle, a theoretical doubling of the target sequence occurs. The whole procedure is carried out in a programmable thermal cycler that precisely controls the temperature at which the steps occur, the length of time the reaction is held at the different temperatures, and the number of cycles. Under ideal conditions, a single copy of a nucleic acid target can be amplified over a billion-fold after 30 cycles, thus allowing amplification from targeted genomic signature with potential detection of etiologic agents down to a single copy.[107–109] Genomic material, DNA or RNA (in the form of cDNA), can be targeted by this method of amplification. Rapid detection methods typically rely on real-time PCR where targeted genomic signatures are amplified via primers and detection accomplished through oligonucleotide probe hybridization. To this end, numerous PCR-based technologies are currently implemented in the clinical setting for diagnosis of infectious agents.

Real-Time Polymerase Chain Reaction

The most important development in rapid identification of biological agents is real-time PCR methods. Although traditional PCR is a powerful analytical tool that launched a revolution in molecular biology, it is difficult to use in clinical and field laboratories. As originally conceived, gene amplification assays can require 5 to 6 hours to complete, not including the sample processing required to remove PCR inhibitors.[110] The improvement of assay time-to-answer came with the development of assay chemistries that allowed the PCR reaction to be monitored during the exponential

amplification phase, that is, real-time (Figure 26-8). In this context, Lee et al and Livak et al developed real-time assays for detection and quantification of fluorescent reporters where fluorescence increase was directly proportional to the amount of PCR product generated in the reaction.[111,112] In this scenario, higher starting copy numbers of the nucleic acid target resulted in earlier amplification where significant increase in fluorescence is observed.

Three main probe-based fluorescence-monitoring systems exist for DNA amplification: (1) hydrolysis probes; (2) hybridization probes; and (3) DNA-binding agents. Hydrolysis probes, most exemplified by TaqMan (Applied Biosystems, Foster City, CA) chemistries, have been the most successful for rapidly identifying biological threats.[108] Numerous assays have been developed against biological threat and infectious agents using these approaches by the DoD, the CDC, and the US Department of Energy.[108,109]

The JBAIDS is the current DoD fielded platform for molecular diagnostic/real-time PCR detection in reference laboratory, combat support hospital, and forward operating settings. This system supports assays primarily in the identification of several biological threat agents for clinical diagnostic application while also supporting assays for biosurveillance screening of biological threats as well as some infectious diseases. FDA-cleared assays for clinical diagnostics include *B anthracis*, *F tularensis*, *Y pestis*, *C burnetii*, and several forms of influenza (H5N1, A, B and A subtyping). Other assays for biosurveillance purposes cover additional biological threat targets, toxins, and foodborne pathogens. These assays can be run in approximately 30 minutes with up to 32 samples per run. With this

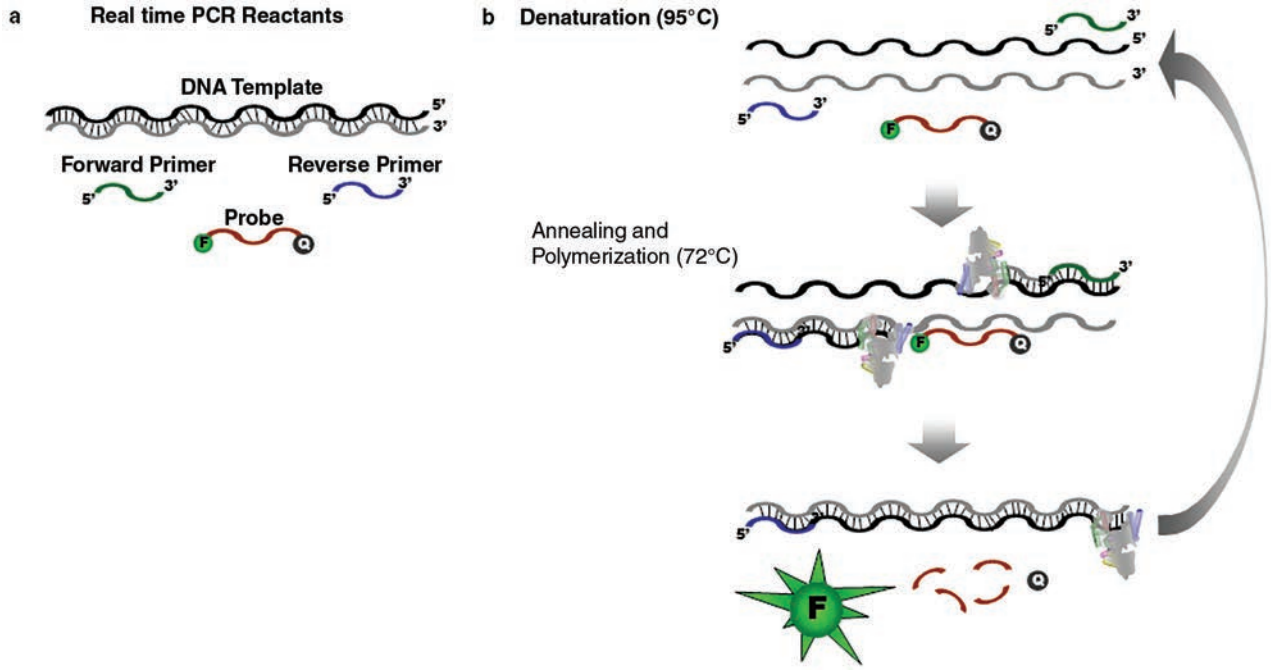


Figure 26-8. Overview of real-time PCR reactants and reaction conditions, generic. (**a**) Real-time PCR reactions (TaqMan probes depicted) consist of the canonical PCR reactants, such as forward and reverse primers as well as a DNA template. In addition to these reactants, real-time PCR contains either a fluorescently labeled probe or intercalating dye that is used to monitor amplicon quantities. In the depicted scenario, a sequence of DNA complementary to target sequence separates a fluorophore (F) and a quencher (Q). Fluorescence from the fluorophore in proximity to the quencher is greatly diminished compared to absence or distal fluorescence. (**b**) Similar to conventional PCR, real-time PCR reactions begin with a denaturing of the DNA template. Reducing the temperature allows amplicon-specific primers to anneal to the target sequence and amplification to begin. In some type of real-time reactions, amplified double-stranded DNA is directly quantified through measurement of DNA intercalating dyes such as SYBR green, which only fluoresces when intercalated. In the instance depicted, the probe anneals to the DNA template in similar fashion to the primers. When DNA polymerase encounters the probe, the enzyme's exonuclease function cleaves the probe liberating the fluorophore. No longer in proximity to the quencher, fluorophore fluorescence can be monitored and then correlated to target sequence concentration. Subsequent cycling and amplification yield progressively more DNA template and, consequently, more fluorophore fluorescence. PCR: polymerase chain reaction

system, a presumptive identification of most biological agents can be completed in 3 hours or less. Although it is an excellent system for detecting biological threat agents, this system suffers from lack of use in the field setting because of the lack of assays for more commonly acquired pathogens that are more routinely seen in the clinical setting. To mitigate this issue, future generations of molecular detection instruments should have regulatory cleared assays for common infectious diseases to make use and maintenance worthwhile.

Next Generation Molecular Diagnostics

The JBAIDS device is currently fielded in DoD medical laboratories, and several of the aforementioned problems exist with this system including the lack of routine usage resulting from limited assay availability and the limited capability to run independent or replicate samples (32 samples per run). To address some of these issues, the Joint Program Executive Office, the office that fielded the JBAIDS, acquired the Biofire FilmArray platform for the NGDS. While the FilmArray (Biofire Diagnostics, Salt Lake City, UT) was chosen as the NGDS device, several other viable diagnostics were considered within source selection, including the Liat Analyzer (IQuum, Marlborough, MA) and the 3M Focus Integrated Cycler (Focus Diagnostics, Cypress, CA). Overall, the FilmArray was chosen based on ease of use, sensitivity, and available FDA-cleared assays for respiratory or other commonly acquired infectious diseases.

FilmArray is an integrated sample prep and multiplex PCR diagnostic platform capable of detecting bacteria and viruses in a single reaction. This system can run FDA-cleared assays for common respiratory organisms or assays for biological threat detection in a pouch-based array, thus providing a routine application for the instrument in a clinical setting. In addition to the respiratory pouch, several other pouches have been evaluated, to include the blood culture and biological threat pouches verifying performance characteristics.[113–116] Up to 48 independent reactions can be run in a single run; however, only a single sample can be run per pouch thereby limiting the throughput of this device. Overall, the system is a simple use instrument using syringe and closed pouch-based system to bead-beat and extracts nucleic acid with downstream application to an array-based set of real-time PCR reactions. Given the low complexity of operation, FDA is evaluating it for a CLIA-waver; however, currently, it is considered a moderate complexity device.

Overall, this system provides an incremental step forward in technology compared to the JBAIDS that it will replace. Assay versatility will be sacrificed for integrated sample processing and clinically applicable assays upon deployment. While these additions to the DoD portfolio will augment current biosurveillance and biological threat detection capabilities, further development is required to truly advance the frontline military diagnostic applications. The current forerunner for filling this capability is next-generation sequencing (NGS) applications.

The Horizon–Agnostic Diagnostic Applications

The current endpoint and desired capability for diagnostics in the DoD is an agnostic molecular platform. All the aforementioned technologies require some a priori knowledge of the organism; for instance, real-time PCR requires sequence information regarding the target of interest to design primers and probe. In addition, in the application of real-time PCR, guidance from medical intelligence, symptomology, or endemic diseases is required because there are limitations to the number of discrete targets and samples that can be queried in a single run. These limitations could be overcome by application of agnostic diagnostic approaches such as NGS pathogen detection strategies.

NGS has many potential benefits over current molecular diagnostic approaches. In terms of agnostic detection, NGS has the capability to sequence an entire genome of an organism, thus obviating the need for specific a priori knowledge of the pathogen. For example, the detection of novel filovirus variants such as Lujo virus was accomplished via NGS discovery.[117] While numerous methodologies have come and gone throughout technology development, current field leaders are Illumina's sequence-by-synthesis (Illumina Inc, San Diego, CA) and PacBio's (Pacific Biosciences, Menlo Park, CA) single molecule real-time sequencing. Each system has advantages and disadvantages. Illumina is the current leader with shorter sequence reads (72–250 bp), but generating significantly more sequence data (>10 GBp). PacBio, however, generates much longer reads (1–10 kb), but has significantly higher error rates. As the field progresses, newer nanopore technologies, such as the MinION (Oxford Nanopore, Oxford, UK), may supplant these current leaders in the near future.

Combinatory approaches between these two technologies have been applied to mitigate independent disadvantages while retaining platform-specific advantages.[118] Numerous lab-derived tests and even 510(k) submissions have cleared the FDA for use in detecting cancer. However, several steps and obstacles require mitigation before these technologies can be applied to regulatory compliant detection of a pathogenic organism. Principal in these issues

include mitigation of high amounts of background host-derived nucleic acid, lack of specificity resulting from agnostic nature, and sensitivity issues. Current efforts within academia and DoD show promise toward mitigating these issues and bringing NGS into the diagnostic toolbox.

BIOSURVEILLANCE AND EMERGING THREATS

The emergence of new biological threats is a particular challenge for the military clinical or field laboratory. In the past, the biological defense research program for diagnostics has focused on agent-specific identification using collections of biological threats in the biological weapons programs of the United States (ended in 1969) and the former Soviet Union.[119,120] However, several critical events have broadened the scope of the biological threat over the past 3 decades. The maturation and proliferation of biotechnology have resulted in several laboratory demonstrations of genetically engineered threats with new, potentially lethal characteristics.[121–125] Jackson et al demonstrated that the virulence of orthopoxviruses was enhanced by the insertion of immunoregulatory genes, such as interleukin-4.[124] In other work, Athamna et al demonstrated the intentional selection of antibiotic-resistant *B anthracis*.[121] Borzenkov, Pomerantsev, and Ashmarin modified *Francisella*, *Brucella*, and *Yersinia* species by inserting beta-endorphin genes.[122,123]

As a result of the proliferation of these biotechniques, public health officials can no longer depend on an adversary choosing any of the 15 to 20 biological threats of past generations, but now must prepare for a future of an infinite number of threats, some of which may have been genetically engineered to enhance virulence or avoid detection. Secondly, the emergence of more virulent and/or infectious strains of naturally occurring infectious diseases has posed significant public health challenges to civilian and military populations. The emergence of the H5N1 and H1N1 variants of influenza is a recent example of the challenge that naturally occurring infectious diseases can present, the latter resulting in a pandemic from 2009 to 2010. These new threats will require the development of identification and diagnostic systems that can be used flexibly to allow early recognition of a unique biological threat, representing one of the next major research and development challenges for the DoD, DHHS, and DHS. The ability to identify and characterize genetically engineered threats or naturally emerging infectious diseases before they negatively affect military and public health is the focus of new initiatives in biosurveillance.

A national effort on biosurveillance was formally initiated on October 18, 2007 in Homeland Security Presidential Directive-21,[126] which defines biosurveillance as the process of active data gathering with appropriate analysis and interpretation of biosphere data that might relate to disease activity and threats to human or animal health—whether infectious, toxic, metabolic, or otherwise, and regardless of intentional or natural origin—to achieve early warning of health threats, early detection of health events, and overall situational awareness of disease activity. The DoD community has accepted biosurveillance as defined above as a working definition, and as synonymous with health surveillance as defined in DoD Directive 6490.02E, Comprehensive Health Surveillance, which establishes policies and assigns responsibility for routine, comprehensive health surveillance of all military service members.[127] The DoD has an extensive health surveillance program for all military personnel, and the Armed Forces Health Surveillance Center executes this effort.[128] However, in addition to human health surveillance, biosurveillance encompasses active data gathering and interpretation of data from the entire biosphere, including animal health surveillance, vector surveillance, and environmental surveillance.[129]

The challenge 7 years removed from the Homeland Security Presidential Directive-21 is accessing, collecting, and interpreting all of the surveillance data that are available in a way that provides actionable information to affect public health. Specific challenges that must be addressed include information sharing, information technology tools to assimilate and analyze data, and algorithms to interpret and report the subset of data that affects public health. Within the confines of biosurveillance, diagnostic testing results are a very small percentage of the health surveillance data, and an even smaller percentage of the biosurveillance data. Therefore, care must be exercised to ensure that diagnostic testing data feed into biosurveillance without allowing the biosurveillance mission to become the critical requirements for diagnostic assay and platform development. Diagnostics must continue to focus on assisting clinicians in making correct medical decisions about the treatment and prognosis of individual patients. The ultimate goal and the significant challenge for the biosurveillance enterprise is translating the identification of a potential public health threat through biosurveillance to a medical countermeasure, such as an in vitro diagnostic test. Doing so in a timely manner will be critical to maintain military readiness and minimize public health impacts.

Ultimately, the success of biosurveillance depends on the tools and technologies available to survey the biological space that affects human health. These tools must move away from agent specific identification, which is currently the foundation of most FDA-cleared in vitro diagnostic tests, to a more agnostic approach. Unlike diagnostic tests, which are typically chosen based on clinical suspicion of a particular disease, biosurveillance platforms must attempt to identify all agent(s) in a particular sample. This identification can be approached through the use of multiple complementary identification technologies or agent agnostic platforms. The service lab component of the NGDS acquisition program will deliver several complementary platforms to OCONUS research laboratories to enhance the DoD's biosurveillance capability. The instruments will include the Applied Biosystems (Foster City, CA) 7500 Fast Dx Real-Time PCR Instrument, the Luminex (Austin, TX) MAGPIX, and the Illumina (San Diego, CA) MiSeq instrument. The 7500 Fast Dx is an FDA-cleared molecular diagnostic device for the detection of nucleic acids by real-time PCR, whereas the MAGPIX is a highly multiplexed combined immunoassay/molecular assay platform for the detection of proteins or nucleic acids. Combined, these instruments could potentially cover the nucleic acid and protein biological space to include identification of viruses, bacteria, and toxins. The critical challenge for these two instruments will be the availability of assays that are capable of extensively surveying the infectious disease space.

In addition, the Illumina MiSeq instrument is a nucleic acid sequencing instrument that may potentially be used as an agnostic approach to agent identification.[130,131] Metagenomic sequencing has become a favored approach to identify all biological components in clinical and environmental samples, and significant investments have been made to stand up genomic sequencing centers within the DoD. The roll out of MiSeq instruments in overseas laboratories is the DoD's attempt to take this capability beyond reference laboratories. Although sequencing has advanced significantly in the past decade, it has proven most useful in samples where the amount of organisms is not limiting, which is often not the case in clinical samples where the concentration of organism is extremely low in relation to the host nucleic acid in the sample. Teasing out the sequences that are significant for biosurveillance and public health purposes is the critical biochemical and bioinformatic challenge for metagenomic sequencing approaches.[132]

Although sequencing provides a wealth of information, sequence data alone does not substitute for the need to propagate and maintain the viable organisms necessary for medical countermeasure development efforts. This capability is critical, especially for unknown or emerging threats, as all vaccine, therapeutic, and diagnostic development will require enough purified agent material to perform the necessary investigations. Overall, a rapid response capability from agent identification to therapeutic delivery to the warfighter requires integration across program areas with logical transition from one capability area to another within DoD. A comprehensive biosurveillance plan will include sample acquisition, identification, and characterization capability that allows for rapid development of medical countermeasures. Transition of the deliverables from biosurveillance should bridge pathogen discovery with diagnostics, animal model development, and vaccine and therapeutic evaluation, thereby shortening the timeline between agent identification and fielding of medical countermeasures. Ultimately, data from biosurveillance efforts must lead to actionable information to respond rapidly with medical countermeasures such as vaccines, therapeutics, and diagnostics.

Ultimately, the information provided by biosurveillance needs to translate into products that can be used in an emergency situation to enhance military readiness and public health. The nation's ability to react to a biological event to minimize casualties and impacts, or biopreparedness, is critical during an emerging outbreak or intentional release of a biological threat agent. The emergence of H1N1 and H5N1 strains of influenza was a valuable lesson for the US government to provide medical countermeasures in a response that included the availability of in vitro diagnostic tests. In 2004, the Project BioShield Act amended the Federal Food and Drug Cosmetic Act (21 USC 360bbb-3; sec 564) to include a process by which non-FDA approved products or off-label uses of approved products could be rapidly fielded in declared emergency situations. Only the Secretary of Defense, Secretary of Homeland Security, or Secretary of Health and Human Services can determine whether an emergency situation meets the criteria established in the act. Once this occurs, the US Secretary of Health and Human Services issues a declaration allowing EUA submissions to the FDA for consideration and potential use. Declared emergencies are not limited to ongoing emergencies, but also include situations that may present a heightened risk for potential attacks or events.

Any potential situation that would pose a significant risk to the public or to US military forces, or has the potential to adversely affect national security could be declared an emergency situation. This process was activated, refined, and used for in vitro diagnostics during the H1N1 pandemic in 2009–2010.[133] The typical

process to use an in vitro diagnostic test during an emergency involves the declaration of emergency, the submission of performance data to the FDA, FDA review, and FDA authorization to use the test under the EUA. One outcome of the H1N1 EUA process for diagnostics was the development of a pre-EUA process to streamline this process. Based on the FDA's H1N1 guidance document,[133] the DoD and FDA worked together to define a process for prepositioning performance data for in vitro diagnostic tests that were not yet FDA cleared but could be invaluable during a declared emergency. By allowing pre-EUA submissions for diagnostic tests, the FDA can review data, request additional data, and make preliminary decisions on utility before an emergency is declared, greatly reducing the time between the declaration of an emergency and the authorization to use the test. Pre-EUA approval does not grant permission to use or market the product under nonemergency conditions, but greatly enhances biopreparedness should a biological threat event occur. The DoD submitted 73 assays for pre-EUA consideration to the FDA in July 2010, and eight assays have been accepted after providing additional performance data on the JBAIDS and Applied Biosystems 7500 FAST DX real-time PCR platforms. The pre-EUA process continues to expand the immediate availability of in vitro diagnostics during a declared emergency, and it adds previously unavailable biopreparedness capability for the DoD and the nation.

Success in responding to emerging or genetically engineered biological threats is dependent on identifying, characterizing, and reducing the health impacts of the threat, which requires a continuum from identification of the threat at the point of presentation (clinically or environmentally) through rapid medical countermeasure deployment. Doing so quickly requires the assimilation of all available biological data, determination of which data are meaningful, and identification of actionable information signifying a threat to public health. These are the underlying goals of biosurveillance. However, the collection of samples, characterization of the threat agent, development or identification of the appropriate countermeasures, and deployment of those countermeasures to be used under regulatory compliance are necessary to achieve the desired end state, thus minimizing the public health and military readiness impacts of emerging and engineered threats.

FUTURE APPROACHES

Early Recognition of the Host Response

Early recognition is critical for the diagnosis and treatment of biological threat agents because of their disease progression, persistence, and lethality (Table 26-9). The host responds to microbial invasion immunologically and also responds to pathological factors expressed by the foreign organism or toxin. Identifying early changes in the host gene response may provide an immediate indication of exposure to an agent and subsequently lead to early identification of the specific agent before the onset of disease. Several biological agents and toxins directly affect components important for innate immunity, such as macrophage or dendritic cell functions or immunomodulator expression.

Host gene responses to biological threat insults can manifest in multiple ways. Studies suggest that the anthrax lethal factor may induce apoptosis in peripheral blood mononuclear cells, inhibit production of proinflammatory cytokines in peripheral blood mononuclear cells, and impair dendritic cells.[134,135] Poxviruses may possess several mechanisms to inhibit innate immunity.[136] Gibb, Norwood, Woollen, and Henchal reported that alveolar macrophages infected with Ebola virus demonstrated transient increases in cytokine and chemokine mRNA levels that were markedly reduced after 2 hours postexposure.[137] Others have shown that Ebola virus infections are characterized by dysregulation of normal host immune responses.[138] However, directly detecting these effects, especially inhibition of cytokine expression, is technically difficult to measure in potentially exposed populations.

New approaches that evaluate the regulation of host genes in microarrays may allow for early disease recognition.[139,140] A complicated picture is emerging that goes beyond dysregulation of genes related to innate immunity. Relman suggested that there are genome-wide responses to pathogenic agents.[141] Mendis identified cDNA fragments that were differentially expressed after 16 hours of in vitro exposure of human peripheral blood mononuclear cells to staphylococcal enterotoxin B.[142] By using custom cDNA microarrays and real-time analysis, these investigators found a unique set of genes associated with staphylococcal enterotoxin B exposure. By 16 hours, there was a convergence of some gene expression responses: many of those genes code for proteins such as proteinases, transcription factors, vascular tone regulators, and respiratory distress. Additional studies are needed to characterize normal baseline parameters from a diverse group of individuals undergoing common physiological responses to the environment, as well as responses to the highest

TABLE 26-9

BIOLOGICAL WARFARE AGENT DISEASE CHARACTERISTICS

Disease	Human-to-Human Transmission	Infective Dose (Aerosol)	Incubation Period	Duration of Illness	Lethality	Persistence of Organism
Anthrax	No	8,000–1,000 spores	1–6 d	3–5 d (fatal if untreated)	High	High
Brucellosis	No	10–100 cells	5–60 d; usually 30–60 d	Weeks to months	Low	High
Glanders	Low	5,000–10,000 cells (NHP)	10–14 d	7–10 d (fatal if untreated)	Moderate to high; >50%	High
Melioidosis	Low	50–80 cells (NHP)	1–21 d; up to years	2–3 d (fatal if untreated)	Moderate	High
Plague	Moderate	500–15,000 cells	1–7 d; usually 2–3 d	1–6 d (fatal if untreated)	High	High
Tularemia	No	10–50 cells	1–21 d; usually 3–6 d	Fatal if untreated	Moderate	High
Q fever	Rare	1–10 cells	7–41 d	2–14 d or longer if not treated	Low	High
Smallpox	High	10–100 organisms	7–17 d; average 12 d	4 weeks	High	High
VEE	Low	10–100 organisms	2–6 d	Days to weeks	Low	Low
Viral hemorrhagic fevers	Moderate	1–10 organisms	4–12 d	Death between 7–16 d	Moderate to high	Low
Botulism	No	0.003 μg/kg for type A	12 h–5 d	Death in 24–72 h; lasts for months if not lethal	High	Low (weeks)
SEB	No	0.0004 μg/kg	3–12 h	Hours	Low	Low
Ricin	No	3–5 μg/kg (mouse LD_{50})	18–24 h	Days	High	High

LD: lethal dose
NHP: nonhuman primate
SEB: staphylococcal enterotoxin B
VEE: Venezuelan equine encephalitis

priority biological agents and toxins in appropriate animal models. Approaches that integrate detection of early host responses with the sensitive detection of biological agent markers can decrease morbidity and mortality by encouraging optimal therapeutic intervention.

SUMMARY

Military clinical and field laboratories play a critical role in the early recognition of biological threats, serving as unique sentinels in CONUS and OCONUS areas for biological threats and emerging infectious diseases. While performing regulatory compliant patient diagnostics for biological threats is difficult in a theater of operation, the fielding of the JBAIDS real-time PCR platform has had some success. The NGDS acquisition program will incrementally improve this capability by providing a highly multiplexed "sample in/answer out" capability for molecular biological threat identification. Although these fielded platforms provide a diagnostic capability in theater, they are not definitive means of identification and are based on targets that are currently well understood. Definitive identification requires orthogonal testing to improve the reliability of rapid diagnostic technologies and reduce risk.

The integration of culture as well as nucleic acid and immunological biomarkers for the identification of biological threat agents is critical to elevate the level of confidence in identifying these high consequence infectious diseases. The network of laboratories available for confirmatory and definitive testing is strong and has improved significantly within the past 5 years. Future technologies will further increase the orthogonal capabilities of diagnostic platforms and strive toward agent agnostic agent identification. The integration of molecular and immunological identification on a single platform using common analytical chemistries may be realized within the next 5 to 10 years, and whole genome metagenomic sequencing holds the promise of identifying all infectious agents in a given sample. These approaches will be critical to accommodate the identification of emerging as well as genetically engineered agents.

Although indications show that these future approaches are making progress, regulatory challenges will occur for diagnostic use of highly multiplexed and sequencing technologies. Fortunately, the FDA has been forward thinking and is currently engaged in identifying the key standards required for both highly multiplexed and whole sample sequencing based approaches for clearance of diagnostics. Biosurveillance initiatives may provide a means to evaluate and improve future platforms that could ultimately transition to diagnostic devices if costs permit. In the meantime, medical diagnostics for biological threat agents will rely on proven technologies that incorporate incremental improvements to simplify and improve the reliability and robustness of diagnostic devices for use throughout the military clinical and field laboratories.

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Chapter 27

MEDICAL COUNTERMEASURES

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INTRODUCTION

BACTERIAL AND RICKETTSIAL DISEASES
- **Anthrax**
- **Tularemia**
- **Plague**
- **Glanders and Melioidosis**
- **Brucellosis**
- **Q Fever**

VIRAL DISEASES
- **Encephalitic New World Alphaviruses**
- **Smallpox**
- **Viral Hemorrhagic Fevers**

TOXINS
- **Botulinum Neurotoxin**
- **Staphylococcal Enterotoxin B**
- **Ricin**

SUMMARY

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INTRODUCTION

Countermeasures against bioterrorism, intended to minimize morbidity and mortality and to prevent or limit the number of secondary infections or intoxications, include (*a*) early identification of the bioterrorism event and persons exposed, (*b*) appropriate decontamination, (*c*) infection control, and (*d*) medical countermeasures. The first three countermeasures are nonmedical and are discussed in other chapters. This chapter covers medical countermeasures, which include interventions such as active immunoprophylaxis (ie, vaccines), passive immunoprophylaxis (immunoglobulins and antitoxins), and chemoprophylaxis (antimicrobial medications). Medical countermeasures may be initiated before an exposure (if individuals can be identified as being at high risk for exposure), after a confirmed exposure event, or after the onset of symptoms in infected individuals.

Because medical countermeasures may be associated with adverse events, the recommendation for their use must be weighed against the risk of exposure and disease. Vaccines, including both investigational vaccines and those approved by the US Food and Drug Administration (FDA), are available for some bioterrorism agents (Table 27-1). In the event of a bioterrorism incident, preexposure vaccination—if safe and available—may modify or eliminate the need for postexposure chemoprophylaxis. However, preexposure vaccination may not be possible or practical in the absence of a known or expected release of a specific bioterrorism agent, particularly with vaccinations that require multiple primary (or priming) doses to achieve immunity or repeated booster doses to maintain it. In these cases, chemoprophylaxis—and, in some cases, immunoprophylaxis—after identifying an exposure or infection may be effective in preventing disease or death (Table 27-2). Any effective plan for countering bioterrorism should address the logistics of maintaining adequate supplies of drugs and vaccines as well as personnel coordinating and dispensing needed supplies to the affected site.

FDA-approved vaccines against anthrax and smallpox are available; however, for many potential bioterrorism agents, only investigational vaccines that were developed and manufactured more than 30 years ago are available. These vaccines have demonstrated efficacy in animal models and safety in at-risk laboratory workers; however, they did not qualify for FDA approval because studies to demonstrate their efficacy in humans were deemed unsafe and unethical. Although they can be obtained under investigational new drug (IND) protocols at limited sites in the United States, these vaccines are in extremely limited supply and some are declining in immunogenicity with age.

Under the FDA Animal Rule instituted in 2002, approval of vaccines, antimicrobials, and other drugs can now be based on demonstration of efficacy in animal models alone if efficacy studies in humans would be unsafe or unethical. This rule provides an opportunity to develop many new and improved vaccines and other medications, with the ultimate goal of FDA licensure. However, drug development generally is a long process. In vaccine development, for example, generally 3 to 5 years is required to identify a potential vaccine candidate and conduct animal studies to test for vaccine immunogenicity and efficacy, followed by 5 years of clinical trials for FDA approval and licensure. FDA vaccine approval then takes from 7 to 10 years, and under the FDA Animal Rule, additional time must be devoted to animal studies to identify correlates of protection. Thus, vaccine replacements are not expected to be available in the near future.

BACTERIAL AND RICKETTSIAL DISEASES

Anthrax

Anthrax is caused by *Bacillus anthracis*, a spore-forming, gram-positive bacillus that can be found in many soil environments worldwide. It occurs in a vegetative state and in a spore state; the spore state, which can remain viable for decades, is the infectious form.[1–3] Ruminants acquire spores by ingesting contaminated soil while grazing. Humans can become infected through skin contact, ingestion, or inhalation of *B anthracis* spores from infected animals or animal products.[3] Anthrax is not transmissible from person to person. Cutaneous anthrax is the most common naturally occurring form of anthrax, and gastrointestinal anthrax is the least common form. Inhalational anthrax, which occurs as a result of exposure to aerosolized spores, is considered the form of disease most likely to result from an act of bioterrorism. Meningitis can occur, as secondary seeding from bacteremia, with any form of anthrax.[4] Because of its virulence, ease of preparation, the potential to aerosolize spores, and the stability and prolonged survival of the spore stage, *B anthracis* is an ideal agent for bioterrorism.[4,5]

TABLE 27-1

VACCINES, VACCINE DOSAGE SCHEDULES, AND CORRELATES OF POSTVACCINATION PROTECTION

Disease	Vaccine (Dose and Route)	Type of Vaccine	Primary Series	Booster Doses	Immunogenicity Criteria
			BACTERIA		
Anthrax	AVA (BioThrax) (0.5 mL IM)	Sterile, acellular filtrate	Months 0, 1, & 6	Months 12 & 18, annually	3 weeks after 3rd dose
Tularemia	NDBR 101[*] (15 punctures, 1 drop [0.06 mL] PC)	Live attenuated	Day 0	None	Take reaction[†] by day 7 after vaccination; day 28 microagglutination titer ≥4-fold rise from prevaccination baseline
Q fever	NDBR 105[*,‡,§] (0.5 mL SC)	Inactivated	Day 0	None	3–5 weeks after vaccination
			VIRUSES		
VEE	TC-83 NDBR 102[*] (0.5 mL SC)	Live attenuated	Day 0	None; boost with C-84 per titer	$PRNT_{80}$ titer ≥1:20
	C-84 TSI-GSD 205[*] (0.5 mL SC)	Inactivated	None[¥]	Initial responders to TC-83 and past recipients of C-84: single boost of C-84 Initial nonresponders to TC-83: boost with C-84	$PRNT_{80}$ titer ≥1:20
WEE	TSI-GSD 210[*,§] (0.5 mL SC)	Inactivated	Days 0, 7, & 28	Mandatory boost: month 6; then as needed per titer	$PRNT_{80}$ titer ≥1:40
EEE	TSI-GSD 104[*] (0.5 mL SC)	Inactivated	Days 0 & 28	Mandatory boost: month 6; before month 6 and after: as needed per titer (0.1 mL ID)	$PRNT_{80}$ titer ≥1:40
Smallpox	ACAM2000 (15 punctures PC)	Cell culture–based live vaccinia virus	Day 0	Every 1, 3, or 10 years[¶]	Take reaction[**] after primary vaccination
Yellow fever	YF-Vax (0.5 mL SC)	Live attenuated	Day 0	Every 10 years	1 month after vaccination
RVF	TSI-GSD 200[*,††] (1 mL SC)	Inactivated	Days 0, 7, & 28	Initial responders[‡‡]: mandatory boost at month 6; then as needed per titer Initial nonresponders[‡‡]: boost within 90 days of low titer	$PRNT_{80}$ titer ≥1:40

(**Table 27-1** *continues*)

Table 27-1 continued

Notes: Vaccines listed are those available in the United States and used (under current or recent protocols) in the Special Immunizations Program at the US Army Medical Research Institute of Infectious Diseases. Vaccines are available elsewhere for Argentine hemorrhagic fever (Candid #1, available in Argentina, which may have cross-protection for Bolivian hemorrhagic fever); Omsk hemorrhagic fever (for which cross-protection is provided from the tickborne encephalitis vaccine FSME-IMMUN); Kyasanur Forest disease (for which a vaccine is available in India); Crimean–Congo hemorrhagic fever (for which a vaccine is available in Bulgaria); and hemorrhagic fever with renal syndrome (Hantavax, which is available in South Korea).
[*]Investigational product.
[†]The take reaction is an erythematous papule, vesicle, and/or eschar, with or without induration, at the vaccination site. Compared with smallpox vaccination (using ACAM2000), tularemia vaccination (using NDBR 101) results in a smaller take reaction with less induration.
[‡]Prevaccination skin test is required before administration of NDBR 105.
[§]Q fever and WEE vaccines are not currently administered in the SIP.
[¥]Used only as booster (if needed per titer) after vaccination with TC-83.
[¶]Booster doses of the smallpox vaccine are recommended every 3–10 years, depending on risk; for example, laboratory researchers working with variola virus (only at CDC) may receive yearly boosters.
[**]The take reaction after smallpox vaccination (using ACAM2000) is a clear vesicle or pustule, approximately 1 cm in diameter.
[††]A live attenuated RVF MP12 vaccine has recently undergone phase 1 and 2 clinical trials.
[‡‡]Titer is assessed 28 days after 3rd primary dose of RVF vaccine. Initial responders ($PRNT_{80}$ titer ≥1:40 after 3rd dose) receive a boost at 6 months with titer assessed 28 days later. For responders to 6-month booster, titer is assessed annually with subsequent booster doses as needed. For initial nonresponders ($PRNT_{80}$ titer <1:40), booster is given within 90 days and titer is assessed 28 days later. For a nonresponse, boost/titer may be repeated up to 4 times before declaring individual a nonresponder. (The month 6 booster does not apply to initial nonresponders.)
AVA: anthrax vaccine adsorbed; EEE: eastern equine encephalitis; ID: intradermal; IM: intramuscular; PC: percutaneous (scarification); $PRNT_{80}$: 80% plaque reduction neutralization titer; RVF: Rift Valley fever; SC: subcutaneous; TSI-GSD: The Salk Institute-Government Services Division; VEE: Venezuelan equine encephalitis; WEE: western equine encephalitis; YF-Vax: yellow fever vaccine
Data sources: (1) Dembek Z, ed. *USAMRIID's Medical Management of Biological Casualties Handbook*. 7th ed. Fort Detrick, MD: US Army Medical Research Institute of Infectious Diseases; 2011. (2) Emergent BioSolutions. *BioThrax* (Anthrax Vaccine Adsorbed) [package insert]. Lansing, MI: Emergent BioDefense Operations Lansing LLC; 2015. (3) Rusnak J, ed. *Occupational Health Manual for Laboratory Exposures to Select (BSL-3 & BSL-4) and Other Biological Agents*. 3rd ed. Fort Detrick, MD: US Army Medical Research Institute of Infectious Diseases; 2011. (4) Rusnak JM, Kortepeter MG, Aldis J, Boudreau E. Experience in the medical management of potential laboratory exposures to agents of bioterrorism on the basis of risk assessment at the United States Army Medical Research Institute of Infectious Diseases (USAMRIID). *J Occ Env Med*. 2004;46:801–811. (5) Sanofi Pasteur Biologics. *ACAM2000*. Cambridge, MA: Sanofi Pasteur Biologics; 2009. (6) Rotz LD, Dotson DA, Damon IK, Becher JA; Advisory Committee on Immunization Practices. Vaccinia (smallpox) vaccine: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2001. *MMWR Recomm Rep*. 2001;50(RR-10):1–25. (7) Monath TP, Cetron MS. Prevention of yellow fever in persons traveling to the tropics. *Clin Infect Dis*. 2002;34:1369–1378. (8) Pittman PR, Liu CT, Cannon TL, et al. Immunogenicity of an inactivated Rift Valley fever vaccine in humans: a 12-year experience. *Vaccine*. 1999;18:181–189.

Vaccination

History of the Anthrax Vaccine. In 1947, a factor isolated from the edema fluid of cutaneous *B anthracis* lesions was found to successfully vaccinate animals.[6] This factor, identified as the protective antigen (PA), was subsequently recovered by incubating *B anthracis* in special culture medium.[7,8] This work led to the development in 1954 of the first anthrax vaccine, which was derived from an alum-precipitated cell-free filtrate of an aerobic culture of *B anthracis*.[9]

This early version of the anthrax vaccine was found to protect small laboratory animals and nonhuman primates (NHPs) from inhalational anthrax.[9] The vaccine also demonstrated protection against cutaneous anthrax infections in employees working in textile mills processing raw imported goat hair. In particular, only three cases of cutaneous anthrax occurred in 379 vaccinated employees, whereas 18 cases of cutaneous anthrax and all five cases of inhalational anthrax occurred in the 754 unvaccinated employees. Based on these results, the vaccine efficacy for anthrax was estimated to be 92.5%. The vaccine failures were found in one person who had received only two doses of vaccine, a second person who had received the initial three doses of vaccine but failed to receive follow-up doses at 6 and 12 months (and was infected at 13 months), and a third person who was within a week of the fourth (month 6) vaccine dose, when titers are known to be lower.[10] Vaccine breakthroughs were uncommon; the few documented cases of cutaneous anthrax occurred in individuals who had not completed the primary series or who were within days of a scheduled primary or booster dose.[10,11]

Anthrax Vaccine Adsorbed (BioThrax). The current FDA-approved anthrax vaccine adsorbed (AVA; see Table 27-1) was derived through improvements of the early alum-precipitated anthrax vaccine, specifically:

- using a *B anthracis* strain that produced a higher fraction of PA;
- growing the culture under microaerophilic instead of aerobic conditions; and
- substituting an aluminum hydroxide adjuvant in place of the aluminum potassium salt adjuvant.[12,13]

TABLE 27-2

TREATMENT AND POSTEXPOSURE PROPHYLAXIS FOR BACTERIAL DISEASES

Disease	Preferred/Recommended Antimicrobials*	Vaccine	Passive Immunotherapy or Antitoxin
Anthrax (inhalational)	***Treatment*** *Meningitis not ruled out*: Initial IV treatment for ≥14 days (or until clinically stable) of ≥3 antibiotics: ciprofloxacin (400 mg IV every 8 h) AND meropenem (2 g IV every 8 h) AND linezolid (600 mg IV every 12 h) Followed by oral treatment as described for PEP, for a total of ≥60 days of antibiotic treatment *Meningitis ruled out*: Initial IV treatment for ≥14 days (or until clinically stable) of ≥2 antibiotics: ciprofloxacin (400 mg IV every 8 h) AND clindamycin (900 mg IV every 8 h) OR linezolid (600 mg IV every 12 h) Followed by oral treatment as described for PEP, for a total of ≥60 days of antibiotic treatment	***Treatment*** Not recommended	***Treatment*** Raxibacumab (40 mg/kg IV) infused over 2 h, 15 min, within 1 h after premedication with diphenhydramine, in combination with antibiotics OR AIGIV (7 vials [420 units] IV), infused at 0.5 mL/min (first 30 min), 1 mL/min incremental infusion rate if tolerated (every 30 min), max infusion rate of 2 mL/min
	PEP† 60 days of ciprofloxacin (500 mg PO every 12 h) OR doxycycline (100 mg PO every 12 h)	***PEP*** AVA‡ (up to 3 doses SC)	***PEP***§ Raxibacumab (40 mg/kg IV), infused over 2 h, 15 min, within 1 h after premedication with diphenhydramine
Tularemia	***Treatment*** 10 days of streptomycin (1 g IM every 12 h) OR gentamicin (5 mg/kg IM or IV daily)	***Treatment*** Not recommended	No passive immunotherapy available
	PEP† 14 days of doxycycline (100 mg PO every 12 h) OR ciprofloxacin (500 mg PO every 12 h)	***PEP*** Not recommended	
Plague	***Treatment*** ≥10 days of streptomycin (1 g IM every 12 h) OR gentamicin (5 mg/kg IM or IV daily) ***PEP***† 7 days of doxycycline (100 mg PO every 12 h) OR ciprofloxacin (500 mg PO every 12 h)	No vaccine available	No passive immunotherapy available
Glanders or melioidosis	***Treatment*** *Intensive therapy, no complications*¥: 10–14 days of ceftazidime (50 mg/kg [up to 2 g] IV every 8 h) *Intensive therapy, with complications*¥: 10–14 days of meropenem (25 mg/kg [up to 1 g] IV every 8 h) *Eradication therapy*¶: ≥12 weeks of TMP-SMZ (PO) OR amoxicillin–clavulanic acid (PO) ***PEP***** 21 days of TMP-SMZ (PO) OR amoxicillin–clavulanic acid (PO)	No vaccine available	No passive immunotherapy available

(**Table 27-2** *continues*)

Table 27-2 continued

Brucellosis	***Treatment*** *Uncomplicated*: 6 weeks of doxycycline (100 mg PO every 12 h) AND 2–3 weeks of streptomycin (15 mg/kg IM daily) *Complicated*: ≥12 weeks of triple-antibiotic therapy (see text and sources cited therein) ***PEP***[††] 21 days of doxycycline (100 mg PO every 12 h) AND rifampin (450–600 mg PO daily)	No vaccine available	No passive immunotherapy available
Q Fever	***Treatment*** *Acute*: 14 days of doxycycline (100 mg PO every 12 h) *Chronic*: Prolonged treatment with doxycycline (100 mg PO every 12 h) AND hydroxychloroquine (200 mg PO every 8 h) (see text and sources cited therein) ***PEP*** Not recommended; monitor for ≥21 days (see text and sources cited therein)	***Treatment*** Not recommended ***PEP*** Not recommended	No passive immunotherapy available

[*]Antimicrobials listed are the first-line therapies or those most often recommended. For alternative antimicrobials, see text and sources cited therein. Dosages are for adults (men and nonpregnant women); for children and for pregnant women, see text and references cited therein.
[†]The antibiotic regimen described for PEP may also be used for treatment in a mass casualty situation where parenteral antibiotics are not available. In the case of plague, treatment in a mass casualty situation would be extended to 10 days.
[‡]Individuals who were not vaccinated before exposure should receive three doses of AVA as PEP; those who previously received one or two doses of AVA should receive two doses of AVA as PEP, and those who received three or more doses of AVA preexposure should receive one dose of AVA as PEP.
[§]Raxibacumab may be used for PEP in high-risk spore exposure cases.
[¥]Initial IV antibiotic therapy for glanders or melioidosis should be continued for a minimum of 10 days and until the patient's condition improves; the intensive IV therapy may need to be extended to ≥4 weeks. For severe neurologic, cutaneous, bone, joint, or prostate infections, TMP-SMZ could be added to the regimen, administered as described for PEP, or see Lipsitz et al (see data source reference 9).
[¶]In the eradication phase of therapy, the dosage of TMP-SMZ depends on weight: for adult >60 kg, give 160 mg/800 mg tablets (2 tablets every 12 h); for adult 40–60 kg, give 80 mg/400 mg tablets (3 tablets every 12 hours); for adult <40 kg, give 160 mg/800 mg tablets (1 tablet every 12 hours) or 80 mg/400 mg tablets (2 tablets every 12 hours). The dosage of amoxicillin–clavulanic acid (which can be given to those who are intolerant of TMP-SMZ or those with strains that are resistant to TMP-SMZ) also depends on weight: for adult ≥60 kg, give 500 mg/125 mg tablets (3 tablets every 8 hours); for adult <60 kg, give 500 mg/125 mg tablets (2 tablets every 8 hours).
[**]This recommendation is based on animal studies and PEP used for possibly exposed laboratory workers; efficacy in preventing disease among exposed humans has not been evaluated. The dosage of TMP-SMZ and amoxicillin–clavulanic acid depends on weight, as described in the previous note.
[††]For high-risk exposures.
AIGIV: Anthrax Immune Globulin Intravenous; AVA: anthrax vaccine adsorbed; IM: intramuscular; IV: intravenous; PEP: postexposure prophylaxis; PO: by mouth; SC: subcutaneous; TMP-SMZ: trimethoprim and sulfamethoxazole, or co-trimoxazole
Data sources: (1) Hendricks KA, Wright ME, Shadomy SV, et al. Centers for Disease Control and Prevention expert panel meetings on prevention and treatment of anthrax in adults. *Emerg Infect Dis*. 2014;20(2). (2) Wright JG, Quinn CP, Shadomy S, Messonnier N; Centers for Disease Control and Prevention. Use of anthrax vaccine in the United States: recommendations of the Advisory Committee on Immunization Practices (ACIP), 2009. *MMWR Recomm Rep*. 2010;59(RR-6):1–30. (3) Dembek Z, ed. *USAMRIID's Medical Management of Biological Casualties Handbook*. 7th ed. Fort Detrick, MD: US Army Medical Research Institute of Infectious Diseases; 2011. (4) Emergent BioSolutions. BioThrax (Anthrax Vaccine Adsorbed) [package insert]. Lansing, MI: Emergent BioDefense Operations Lansing LLC; 2015. (5) GlaxoSmithKline. *Raxibacumab*. Research Triangle Park, NC: GlaxoSmithKline; 2014. (6) Rusnak J, ed. *Occupational Health Manual for Laboratory Exposures to Select (BSL-3 & BSL-4) and Other Biological Agents*. 3rd ed. Fort Detrick, MD: US Army Medical Research Institute of Infectious Diseases; 2011. (7) Dennis DT, Inglesby TV, Henderson DA, et al. Tularemia as a biological weapon: medical and public health management. *JAMA*. 2001;285:2763–2773. (8) Inglesby TV, Dennis DT, Henderson DA, et al. Plague as a biological weapon: medical and public health management. *JAMA*. 2000;283:2281–2290. (9) Lipsitz R, Garges S, Aurigemma R, et al. Workshop on treatment of and postexposure prophylaxis for *Burkholderia pseudomallei* and *B mallei* infection, 2010. *Emerg Infect Dis*. 2012;18:e2. (10) Ariza J, Bosilkovski M, Cascio A, et al. Perspectives for

(**Table 27-2** *continues*)

Table 27-2 continued

the treatment of brucellosis in the 21st century: the Ioannina recommendations. *PLoS Med*. 2007;4:e317. (11) Centers for Disease Control and Prevention. Laboratory-acquired brucellosis—Indiana and Minnesota, 2006. *MMWR Morb Mortal Wkly Rep*. 2008;57:39–42. (12) Anderson A, Bijlmer H, Fournier P-E, et al. Diagnosis and management of Q fever–United States, 2013: recommendations from CDC and the Q fever working group. *MMWR Recomm Rep*. 2013;62(RR-03):1–30.

Originally produced by the Michigan Department of Public Health, AVA is manufactured by Emergent BioDefense Operations Lansing LLC (Lansing, MI) and marketed under the name BioThrax. It is licensed for preexposure use (and postexposure use, see below) in adults aged 18 to 65.[14,15]

AVA is derived from a sterile cell-free filtrate (with no dead or live bacteria) from cultures of an avirulent, nonencapsulated strain of *B anthracis* (toxinogenic, nonencapsulated v770-np1-R) that produces predominantly PA in the relative absence of other toxin components, such as lethal factor or edema factor.[12,16] The filtrate used to produce AVA is adsorbed to aluminum hydroxide (Amphogel [Wyeth Laboratories, Madison, NJ]) as an adjuvant and contains PA, formaldehyde, and benzethonium chloride, with trace lethal factor and edema factor components.[16] Currently, AVA is given as an intramuscular injection (in the lower two-thirds of the deltoid muscle) of 0.5 mL at months 0, 1, and 6 (the primary series) with boosters at months 12 and 18 followed by yearly boosters as long as the individual remains at risk for anthrax infection.[15]

Both the earlier alum-precipitated vaccine and AVA have demonstrated efficacy against aerosol challenge in animal models.[8,9,13,17–23] In their summary of several NHP studies, Friedlander et al[24] noted that, of 55 monkeys given two doses of AVA, 52 (95%) survived lethal aerosol challenge without antibiotics. More recently, the Centers for Disease Control and Prevention (CDC) showed that three doses of AVA, administered intramuscularly at months 0, 1, and 6, protected rhesus macaques against inhalational anthrax for up to 4 years.[23]

Evidence suggests that both humoral and cellular immune responses against PA are critical to protection against disease after exposure.[12,17,18] Vaccinating rhesus macaques with one dose of AVA elicited anti-PA immunoglobulin (Ig) M titers peaking at 2 weeks after vaccination, IgG titers peaking at 4 to 5 weeks, and PA-specific lymphocyte proliferation present at 5 weeks.[19] In the CDC study, survival of macaques was correlated with cellular and humoral immune responses measured during and after administration of the first three doses of the primary series.[23] After three doses of AVA, 83% to 100% of human vaccinees seroconvert.[25–27] A missed dose of AVA does not necessitate restarting the primary series as recent evidence has demonstrated that the immune response among vaccinees whose month 6 dose is delayed by as much as 7 years is noninferior to that of individuals receiving this dose on schedule.[28] However, the correlation between protection against anthrax infection and a specific antibody titer in humans is not yet clear.[17]

Vaccine Adverse Events. Adverse reactions to AVA are rarely severe when they occur. Adverse reactions in 6,985 persons who received a total of 16,435 doses of AVA, administered subcutaneously according to the original dosing schedule (at weeks 0, 2, and 4 and months 6, 12, and 18 followed by annual boosters), were primarily local reactions (edema or induration). These reactions were severe (>12 cm) in fewer than 1% of vaccinations, moderate (3–12 cm) in 3% of vaccinations, and mild (<3 cm) in 20% of vaccinations. Systemic reactions were uncommon, occurring in fewer than 0.06% of vaccinees, and included fever, chills, body aches, or nausea.[29] After the distribution of around 2 million doses of vaccine, according to the original dosing schedule and route, to more than 500,000 military personnel, data from the Vaccine Adverse Event Reporting System (VAERS) from 1998 to 2001 showed approximately 1,841 reports describing 3,991 adverse events following AVA vaccination. The most frequently reported events were injection site inflammation (752), "flu-like symptoms" (254), systemic rash (251), malaise/fatigue (236), arthralgia (229), and headache (196). Only 96 events (2%) were serious; of those, only 19 were deemed possibly, probably, or certainly related to the receipt of AVA, including anaphylaxis reported in two cases. Three additional cases of anaphylactic-like reactions were reported, but were not deemed serious.[30]

With intramuscular injections administered according to the current dosing schedule (which extends the interval between the first and second primary doses, such that doses are given at months 0, 1, 6, 12, and 18 followed by annual boosters), the incidence of injection site (but not systemic) reactions has been reduced compared with the original AVA dosing schedule and route, with immune responses that are, by month 7, noninferior to those elicited using the original route and schedule.[27,31,32]

Women are more likely than men to experience adverse reactions, particularly certain local reactions, after AVA administration. In an anthrax vaccine study

conducted in laboratory workers and maintenance personnel at the US Army Medical Research Institute of Infectious Diseases (USAMRIID) over 25 years, female vaccinees were more likely than male vaccinees to have injection site reactions, including edema and lymphadenopathy, after subcutaneous injections of AVA.[33] A recent phase 4 clinical trial comparing the subcutaneous and intramuscular routes of administration found that female vaccinees are more likely than male vaccinees to experience injection site reactions. Although this was true regardless of the route of administration, adverse reactions in vaccinees of both genders were reduced with intramuscular administration. For example, erythema occurred in 34% (intramuscular injection of AVA) vs 76% (subcutaneous injection of AVA) of female vaccinees and in 24% vs 48% of male vaccinees; induration occurred in 14% (intramuscular) vs 43% (subcutaneous) of female vaccinees and 10% vs 24% of male vaccinees; and edema occurred in 19% vs 36% of female vaccinees and in 14% vs 27% of male vaccinees.[27] Other factors also appear to predict reactogenicity; in particular, obese women are more likely than those of normal weight to experience local reactions, at least with subcutaneous administration.[34,35] Race may also be associated with reactogenicity.[35]

A 2002 report by the Institute of Medicine's Committee to Assess the Safety and Efficacy of the Anthrax Vaccine found that immediate, short-term adverse effects occur after AVA administration at rates similar to those associated with other licensed vaccines. Rare but serious problems have been reported, but this is true of other licensed vaccines as well. No evidence suggests that AVA causes long-term health problems; however, as with all vaccines, data regarding potential long-term effects are limited.[35a]

Protocols for managing adverse events associated with AVA administration have not yet been evaluated in randomized trials. However, individuals with local adverse events may be managed with ibuprofen or acetaminophen for pain, second-generation antihistamines if localized itching is a dominant feature, and ice packs for severe swelling extending below the elbow.[36]

In persons who have experienced an anaphylactic reaction to the vaccine or any of the vaccine components, subsequent anthrax vaccine doses are contraindicated. AVA is also contraindicated in persons with a history of anthrax infection because of previous observations of an increase in severe adverse events.[37] The vaccine may be given in pregnancy only if the benefit outweighs the risk.

Other Anthrax Vaccines. Another PA-based anthrax vaccine, anthrax vaccine precipitated (AVP), is made by alum precipitation of a cell-free culture filtrate of a derivative of the attenuated *B anthracis* Sterne strain. This vaccine, which is currently licensed in the United Kingdom, is administered as a primary series of four vaccinations at weeks 0, 3, 6, and 32 followed by annual boosters.[21,38,39]

A live attenuated anthrax vaccine (LAAV), which is produced in Russia, is licensed for use in humans in Georgia and Azerbaijan; it is unclear whether the vaccine is licensed elsewhere, such as other former Soviet Union republics or China. LAAV is reported to be protective in mass field trials, in which anthrax occurred less commonly in vaccinated persons (2.1 cases per 100,000 persons), a risk reduction of cutaneous anthrax by a factor of 5.4 in the 18 months after vaccination.[40–42]

Vaccine Research. Although AVA and AVP are safe and effective, the lengthy dosing regimens for these vaccines are onerous, even with the recent dose reduction for AVA,[43] and do not lend themselves to use for rapid prophylactic protection of military personnel deploying to high-risk regions. Ongoing research to improve the current vaccines includes efforts to enhance their efficacy by combining them with alternative adjuvants[44] and by extending the intervals between some doses.[28,39]

The ability to prepare purified components of anthrax toxin by recombinant technology has presented the possibility of new anthrax vaccines. For example, a phase 1 clinical trial has found that an anthrax vaccine using recombinant *Escherichia coli*–derived *B anthracis* PA was safe and well tolerated and elicited a robust humoral and cellular response after two doses.[45] Other new PA-based vaccine candidates combine PA with other components of *B anthracis*, such as formaldehyde-inactivated spores, or use alternative delivery systems, such as intranasal or transdermal routes. DNA vaccines, in which immunogen-encoding genetic material is introduced into a host cell, may provide longer-lasting immunity. Such vaccines have only been explored in animal models.[43,46]

Passive Immunotherapy

The passive administration of polyclonal or monoclonal antibodies directed against PA or other *B anthracis* components is receiving attention as potential postexposure prophylaxis (PEP) or treatment. The recombinant, fully humanized monoclonal antibody raxibacumab and the polyclonal antiserum anthrax immune globulin intravenous (human) (AIGIV; marketed as Anthrasil by Emergent BioSolutions, Lansing, MI), which is derived from the plasma of AVA-vaccinated individuals, both have shown promise in animal studies of efficacy and appear to be safe and well tolerated in humans.[3] This approach to neutralizing anthrax

toxins may be especially effective when used in combination with antibiotic treatment or vaccination.[46–48] Recently, raxibacumab obtained FDA approval for the treatment—combined with antibiotics—of adults and children with inhalational anthrax. Raxibacumab may also be used as PEP for possible aerosol exposure to *B anthracis* when other options are not available or appropriate.[49,50] In March 2015, AIGIV received FDA approval for the treatment of inhalational anthrax, in combination with appropriate antibacterial drugs. MDX-1303 (marketed as Valortim by PharmAthene, Annapolis, MD), is another fully human monoclonal antibody being developed for therapeutic and PEP uses.

Antibiotic Agents

Antibiotics are effective against only the vegetative form of *B anthracis*, not the spore form. In the NHP model of inhalational anthrax, spores have survived in lung tissue for months (with 15%–20% spore survival at 42 days, 2% at 50 days, <1% at 75 days, and trace spores present at 100 days) in a dormant state.[37,51,52] The 1979 outbreak of inhalational anthrax in humans after an accidental release of spores from a Soviet biological weapons production facility (the Sverdlovsk outbreak) further supports the notion that lethal spores can persist in lung tissue after the initial exposure because cases of human anthrax developed as late as 43 days after the release.[53] For this reason, a 60-day course of antibiotics is recommended both for the treatment of inhalational anthrax and as prophylaxis after inhalational exposure (but before symptom onset) in vaccinated and unvaccinated individuals. Prolonged spore survival has not been observed for other routes of exposure.

Ciprofloxacin, doxycycline, levofloxacin, and penicillin G procaine have been FDA approved for treatment of inhalational anthrax and for PEP.[16,37,51,54–56] Ciprofloxacin, doxycycline, and penicillin have reduced the incidence or progression of disease in NHPs after aerosol exposure to *B anthracis*.[37,51,55,57] In macaques exposed to 240,000 to 560,000 anthrax spores (8 median lethal doses), postexposure antibiotic prophylaxis with 30 days of penicillin, doxycyline, or ciprofloxacin resulted in survival of 7 of 10, 9 of 10, and 8 of 9 monkeys, respectively. All animals survived while on prophylaxis, but three monkeys treated with penicillin died between days 39 and 50 postexposure, one monkey treated with doxycycline died on day 58 postexposure, and one monkey treated with ciprofloxacin died on day 36 postexposure.[51] These deaths were attributed to the germination of spores that had persisted in lung tissue after inhalational exposure.

Among human patients with inhalational anthrax between 1900 and 2005, Holty et al[58] found that mortality was significantly lower for those who received (*a*) multidrug antibiotic regimens, (*b*) treatment (with antibiotics or anthrax antiserum) during the prodromal phase of the illness, or (*c*) pleural fluid drainage. Compared with historical cases, patients who were treated for inhalational anthrax during the fall 2001 bioterrorism incident at the Brentwood Post Office and Senate office building in the United States were more likely to have had therapy initiated during the prodromal phase of the disease, to have received several antibiotics, or to have had pleural fluid drainage. These patients were also less likely to die (45% vs 92%).

Adverse events associated with the prolonged, 60-day, antibiotic prophylaxis regimen have had a significant impact on compliance. Overall compliance was reported to be around 44% among the 10,000 persons at six eastern US sites in the 2001 incident for whom the regimen (using ciprofloxacin, doxycycline, or amoxicillin) was recommended.[59] At least one adverse event was reported by 45% and 77% (at day 10 and day 30, respectively) of the individuals receiving PEP most recently with ciprofloxacin. Among those receiving PEP most recently with doxycycline, 49% (day 10) and 71% (day 30) reported experiencing at least one adverse event. Adverse events at day 30 for ciprofloxacin and doxycycline were primarily gastrointestinal symptoms, including nausea, vomiting, diarrhea, abdominal pain, or heartburn (42% and 49% for ciprofloxacin and doxycycline, respectively); fainting, dizziness, light-headedness, or seizures (23% and 18%); rash, hives, or itchy skin (14% and 14%); and joint problems (25% and 16%). Among the 2,631 individuals who took at least one dose of an antibiotic as PEP but stopped taking the drug before completing the full 60-day course, reasons cited for early discontinuation included adverse events (43%), fear of long-term side effects from PEP (7%), and a perception of having a low risk for anthrax (25%).[59] Other adverse events that can occur with quinolones but were not reported in this survey include headache, tremors, restlessness, confusion, and Achilles tendon rupture.

Because of the long-term persistence of spore forms of *B anthracis* in lung tissue after an inhalational exposure, antibiotic prophylaxis combined with vaccination would provide more prolonged protection than postexposure antibiotic prophylaxis alone.[3,37,52] Several studies in rabbits and NHPs have demonstrated that PEP that combines antimicrobial treatment with two or three doses of AVA is protective.[51,60] However, postexposure vaccination without concomitant antimicrobial treatment will not prevent disease from inhalational anthrax.

Some strains of *B anthracis* have shown resistance to certain broad-spectrum antibiotics, such as penicillin, trimethoprim combined with sulfamethoxazole (TMP-SMZ, also called co-trimoxazole), and cefuroxime. Because *B anthracis* strains could be engineered to be resistant to multiple antibiotics, including the current first-line treatments, more selective antibiotic drugs (eg, triclosan derivatives and oligochlorophens) and drug targets (eg, the bacterial cell division protein FtsZ) are being studied.[61–63]

Postevent Countermeasures: Current Options

Treatment. The recommended treatment for inhalational anthrax—and for other forms of anthrax with systemic involvement—varies somewhat depending on whether meningitis has been ruled out. According to the CDC's guidelines,[3] if meningitis has not been ruled out, patients with inhalational anthrax (adults including pregnant women and children)—whether vaccinated or not—should be treated initially with a combination of at least three antimicrobial drugs—all with good central nervous system (CNS) penetration—administered intravenously. This treatment should be continued for at least 2 to 3 weeks or until the patient is clinically stable, whichever is longer.[3,64,65] The drug combination should include at least one bactericidal agent—although Bradley et al[64] recommend two bactericidal agents for children—and at least one protein synthesis inhibitor (see Table 27-2). The preferred bactericidal agents for adults and children are ciprofloxacin (with levofloxacin or moxifloxacin as alternatives) and meropenem (with imipenem and doripenem as alternatives); for penicillin-susceptible strains, penicillin G or ampicillin can serve as the second bactericidal agent.[3,64,65] For pregnant women, ciprofloxacin is the preferred bactericidal agent.[65] The preferred protein synthesis inhibitor for adults (including pregnant women) and children is linezolid (with clindamycin, rifampin, or chloramphenicol as alternatives).[3,64,65] For pregnant women, at least one antibiotic in the combination should be able to cross the placenta (eg, ciprofloxacin, levofloxacin, amoxicillin, or penicillin).[65]

If meningitis has been ruled out, the CDC's guidelines indicate that the initial intravenous treatment for patients of all age groups (including pregnant women) should consist of a combination of at least two antimicrobial drugs, administered intravenously, for at least 2 weeks or until the patient is stable. In this case, CNS penetration is not crucial, but again, at least one agent should be bactericidal and at least one should be a protein synthesis inhibitor. Ciprofloxacin remains the first-choice bactericidal agent for adults (including pregnant women) and children, though penicillin G or ampicillin could be used if the strain is susceptible. Alternative bactericidal agents include meropenem, levofloxacin, imipenem, and vancomycin[3,64] for adults. Hendricks et al[3] and Meaney-Delman et al[65] additionally include moxifloxacin and doripenem as alternative bactericidal agents. Clindamycin or linezolid are the first-choice protein synthesis inhibitors for patients of all ages in whom meningitis has been ruled out,[3,64,65] though Bradley et al[64] indicate that clindamycin is preferred over linezolid for children. Alternative protein synthesis inhibitors include doxycycline and rifampin.[3,64,65] For pregnant women, at least one antibiotic in the combination should be able to cross the placenta.[65]

The CDC's guidelines recommend adding AIGIV or raxibacumab, when available, to combination antibiotic therapy for adults (including pregnant women) and children with inhalational anthrax or other forms of anthrax with systemic involvement.[3,64,65]

For adults, whether or not meningitis has been ruled out, intravenous combination therapy should be followed by oral administration of a single antibiotic, as described below for PEP, such that antibiotic treatment continues for a total of at least 60 days.[3,64,65] For children, Bradley et al[64] recommend follow-up therapy that is essentially the same as that for adults, except that a combination of two antibiotics—one bactericidal agent and one protein synthesis inhibitor—should be used for children who are slower to recover or who, at the end of the initial intravenous treatment, continue to show signs of infection. Ciprofloxacin (with levofloxacin as an alternative) is the preferred bactericidal agent unless the strain is susceptible to penicillins, in which case, amoxicillin (or penicillin VK) would be preferred. Clindamycin is the preferred protein synthesis inhibitor, with doxycycline or linezolid as alternatives.[64]

Postexposure Prophylaxis. Any individual with known or suspected exposure (of greater than negligible risk) to aerosolized *B anthracis*, whether vaccinated or not, should receive antibiotic prophylaxis starting as soon as possible and continuing until *B anthracis* exposure has been excluded (see Table 27-2). If exposure is confirmed or cannot be excluded, PEP should continue for at least 60 days (to clear germinating spores). Prophylaxis should be initiated without delay for the greatest chance of success, but the specific drugs chosen should be subsequently modified if necessary based on the results of strain sensitivity testing.[3,4,14,64–66]

First-line drugs for PEP (prior to symptom onset) for adults (including pregnant women) and children are ciprofloxacin or doxycycline, administered

orally[3,64,65]; ciprofloxacin is preferred over doxycycline for pregnant women.[65] Alternatives, if first-line drugs are contraindicated, not tolerated, or unavailable, include levofloxacin, moxifloxacin, and clindamycin. Amoxicillin and penicillin VK are also acceptable alternatives if the *B anthracis* strain is susceptible to penicillins.[3,4,14,66] Although permanent dental staining has been associated with use of tetracyclines in young children, Bradley et al[64] suggest that doxycycline may be less likely than older tetracyclines to have this effect and argue that such risks are outweighed by the benefits of its use in the event of possible exposure to anthrax. Similarly, Bradley et al[64] suggest that the potential risk of cartilage toxicity from ciprofloxacin is outweighed by the benefits of its use as PEP in this context. If the strain of *B anthracis* involved is found to be susceptible to penicillins, amoxicillin would be the first choice for children.[64]

As of November 2015, AVA is licensed by FDA for PEP—when used in conjunction with recommended antibiotics—in adults aged 18 to 65 years who have been exposed to aerosolized spores.[15] The recommended BioThrax PEP vaccination schedule for those not previously vaccinated is 0.5 mL subcutaneously at weeks 0 (diagnosis), 2, and 4. Individuals who received one or two doses of AVA before exposure should receive two doses of AVA (at weeks 0 and 2). Those who received three or more doses of AVA before exposure should receive a single booster as soon as possible after exposure.[3,15] The CDC additionally recommends the use of AVA and antimicrobial therapy in individuals of all ages, including children and pregnant women, after an aerosol exposure,[3,4,64,65] although Bradley et al[64] recommend delaying administration of AVA for newborns until 6 weeks of age.

Tularemia

Francisella tularensis is a highly infectious, aerobic, non-spore-forming, gram-negative coccobacillus responsible for serious illness and occasionally death.[67,68] Humans can acquire tularemia through (*a*) contact of skin or mucous membranes with the tissues or body secretions of infected animals, (*b*) bites of infected arthropods, (*c*) ingestion of contaminated food or water, or (*d*) inhalation of aerosolized agent from infected animal secretions.[67] Person-to-person transmission of tularemia—although theoretically possible and reported at least once[69]—is considered rare and unlikely.[67]

Most patients with naturally occurring tularemia present with the ulceroglandular form of the disease (generally from intradermal exposure), and up to about one-quarter of patients have typhoidal tularemia (usually resulting from inhalation of infectious aerosols but occasionally from other exposure routes).[14,66,70] Other presentations of tularemia include glandular, oculoglandular, oropharyngeal, and pneumonic (from inhalation or from hematogenous spread from other sites).[66] Pneumonic tularemia and typhoidal tularemia with pulmonary symptoms are the most lethal forms of the disease, yet antibiotics have greatly reduced mortality from all forms of tularemia.[66,70] Disease severity varies by subspecies (or biovar); in particular, two subspecies—*F tularensis* subspecies *tularensis* (type A) and *F tularensis* subspecies *holarctica* (type B)—cause the majority of human disease.[71] Outbreaks of tularemia—particularly inhalational tularemia—in nonendemic areas should alert officials to the possibility of a bioterrorism event.

Vaccination

Investigational Live Tularemia Vaccine. No licensed vaccine protecting against tularemia is available. Vaccination of at-risk laboratory personnel with an inactivated phenolized tularemia vaccine (Foshay vaccine) during the US offensive biological warfare program at Fort Detrick (before 1959) ameliorated disease, but did not prevent infection.[72–74] A sample of the Soviet live *F tularensis* subspecies *holaractica* vaccine (known as strain 15), which the Soviet Union used to vaccinate millions of persons during epidemics of type B tularemia beginning in the 1930s, was made available to Fort Detrick in 1956.[71,73] Both a gray-variant and a blue-variant colony were cultivated from this vaccine (colonies appeared blue when illuminated with oblique light under a dissecting microscope). The blue-variant colony proved to be both more virulent and more immunogenic than the gray-variant colony. To improve protection against the virulent *F tularensis* subspecies *tularensis* SCHU S4 strain, the blue-variant colony was passaged through white mice. These passages resulted in the derivative vaccine strain known as the live vaccine strain (LVS). The strain was used to prepare a lyophilized preparation known as the live tularemia vaccine, which was composed of 99% blue-variant and 1% gray-variant colonies.

During the 27 years of the US offensive biological warfare program at Fort Detrick, tularemia was the most common laboratory-acquired infection. Most of the 161 cases were acquired from aerosol exposures.[11] Beginning in 1959, the live attenuated tularemia vaccine—prepared from, and known as, LVS—was administered to the program's at-risk laboratory personnel until the program closed in 1969 (Figure 27-1).[72] After vaccination using LVS was instituted, the incidence of typhoidal/pneumonic tularemia decreased from 5.7 to 0.27 cases per 1,000 at-risk employee-years. Although



Figure 27-1. Live attenuated NDBR 101 tularemia vaccine. Vaccination of at-risk laboratory workers, beginning in 1959, resulted in a decreased incidence of typhoidal tularemia from 5.7 to 0.27 cases per 1,000 at-risk employee-years, and ameliorated symptoms from ulceroglandular tularemia. The vaccine is administered by scarification with 15 to 30 pricks on the forearm, using a bifurcated needle.

no decrease in ulceroglandular tularemia was noted, the vaccine did ameliorate symptoms from ulceroglandular tularemia, and, unlike those who were unvaccinated before the start of the vaccination program, vaccinated persons did not require hospitalization.[11] The occurrence of ulceroglandular tularemia in vaccinated persons was consistent with the observation that, although natural disease confers immunity to subsequent infections of typhoidal/pneumonic tularemia, it fails to protect against ulceroglandular tularemia. In 1961, commercial production of LVS was initiated by the National Drug Company (Swiftwater, PA), under contract to the US Army Medical Research and Materiel Command. This vaccine, designated NDBR 101, continues to be given to at-risk laboratory workers at USAMRIID under an IND protocol (see Table 27-1).

The live attenuated NDBR 101 tularemia vaccine is supplied as a lyophilized preparation and reconstituted with sterile water before use, resulting in approximately 7×10^8 viable organisms per mL. The vaccine is administered by scarification, with 15 to 30 pricks to the ulnar side of the forearm using a bifurcated needle and a droplet (approximately 0.1 mL) of the vaccine. The individual is examined after vaccination for a take reaction, similar to the examination done after smallpox vaccination. A take with tularemia vaccine is defined as the development of an erythematous papule, vesicle, and/or eschar with or without induration at the vaccination site; however, the postvaccination skin lesion is markedly smaller and has less induration than is generally seen in vaccinia vaccinations. Although a take is related to immunity, its exact correlation has not yet been determined (Figure 27-2).

Protective immunity against *F tularensis* is considered to be primarily cell mediated. Cell-mediated immunity has been correlated with a protective effect, and lack of cell-mediated immunity has been correlated with decreased protection.[75,76] Cell-mediated immune responses occur within 1 to 4 weeks after naturally occurring infection or after LVS vaccination and reportedly last 1 to 3 decades.[75,77–85] Absolute levels of agglutinating antibodies in persons vaccinated with aerosolized LVS could not be correlated with immunity, although the presence of agglutination antibodies in vaccinated persons suggested that they were more resistant to infection than those in the unvaccinated control group.[86] A similar experience was observed in studies of the inactivated Foshay tularemia vaccine,



Figure 27-2. "Take" from the live attenuated NDBR 101 tularemia vaccine at day 7 postvaccination.
Photograph: Courtesy of Special Immunizations Program, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

in which antibodies were induced by the vaccine but were not protective against tularemia.[72,74] Although nearly all vaccinees develop a humoral response, with microagglutination titers appearing between 2 and 4 weeks postvaccination,[75,82,87] a correlation could not be demonstrated between antibody titers and the magnitude of lymphocyte proliferative responses.[76,84,88,89] An explanation for this discrepancy may be that the two types of immune responses are directed toward different antigenic determinants of the organism, with a protein determinant responsible for the cell-mediated immune response and a carbohydrate determinant causing the humoral response.[88]

NDBR 101 has not been licensed in the United States because of drawbacks, including the following:

- the vaccine's uncertain history;
- its unclear mechanism of attenuation (and therefore risk of reversion to virulence); and
- the inconsistency across lots in the proportion of blue and gray colonies present.[71,90,91]

In experimental aerosol exposures of human volunteers, this vaccine protected only 71% to 83% of individuals from inhalational tularemia.[92,93] Because of the short incubation period of tularemia, postexposure use of this vaccine is not recommended. NDBR 101 is recommended for laboratory personnel working with *F tularensis*.[67]

The local skin lesion after vaccination (the take) is an expected occurrence and may result in the formation of a small scar. At the site of inoculation, a slightly raised erythematous lesion appears, which may become papular or vesicular and then forms a scab lasting approximately 2 to 3 weeks. Local axillary lymphadenopathy is reported in 20% to 36% of vaccinees. Systemic reactions are uncommon (<1%) and may include mild fever, malaise, headache, myalgias, arthralgias, and nausea. Mild elevation of liver function tests was noted in some vaccinees but was not determined to be vaccine related. The main contraindications of the vaccine are prior tularemia infection, immunodeficiency, liver disease, and pregnancy.

Vaccine Research

Research is ongoing to develop a new LVS tularemia vaccine (using NDBR 101 as starting material) as well as subunit vaccines against tularemia.[94] Improvements to the LVS vaccine have included efforts to produce LVS under current good manufacturing practice conditions. Subunit vaccines have shown some promise with newly developed adjuvants, such as immune stimulating complexes and CpGs.[91] Live attenuated mutant strains of *F tularensis* derived from LVS and SCHU S4 are also being studied for their attenuation and protection against lethal *F tularensis* challenge.[71]

Antibiotic Agents

The early initiation and adequate duration of therapy are key to the successful treatment of patients with tularemia.[95] Streptomycin, the traditional treatment choice, has proven to be highly efficacious with a low risk of relapse, based on documented cases.[96] However, this aminoglycoside carries the risk of side effects, such as vestibular toxicity and nephrotoxicity, and is often unavailable. Gentamicin, which has also proven efficacious in documented cases, appears to be an acceptable substitute. Fluoroquinolones, such as ciprofloxacin, have shown promise as therapy based on evidence in mice and from use in a human outbreak.[70,95] Fluoroquinolones are highly active against *F tularensis* types A and B in vitro, and evidence from animal studies as well as human cases suggest that ciprofloxacin, levofloxacin, and moxifloxacin are likely to be effective.[95,97] Ciprofloxacin, in particular, has generally had high efficacy with few side effects in adults and children.[67,96] Treatment with 15 days of tetracycline (2 g daily, beginning within 48 hours of symptom onset) was effective in human volunteers exposed to an aerosol challenge of *F tularensis*. Reducing the treatment duration to 10 days or reducing the dose to 1 g daily increased the incidence of relapse.[98] However, tetracyclines may be associated with a greater risk of treatment failure or relapse.[67]

In humans challenged intradermally with an inoculation of *F tularensis*, 5 days of streptomycin, which is bactericidal at concentrations achieved in humans,[95] successfully prevented tularemia.[99] However, neither chloramphenicol nor tetracycline given in a 5-day course was effective as PEP.[99] Tetracycline—given as a 1-g dose twice daily for 14 days, starting within 24 hours of exposure—prevented the development of tularemia in eight humans exposed to aerosols of 25,000 *F tularensis* SCHU S4 spores. However, decreasing the tetracycline dose to only 1 g daily was not as effective in preventing tularemia, with 2 of 10 persons becoming ill. The failure of once-daily tetracycline to prevent tularemia may be caused by considerable fluctuations in tissue levels of the antibiotic, as demonstrated in monkeys given once daily tetracycline, in which the antibiotic ameliorated symptoms but did not prevent tularemia.[98] *F tularensis* is an intracellular pathogen that is cleared slowly from host cells, even in the presence of

bacteriostatic antibiotics. Even in high concentrations, tetracyclines and chloramphenicol merely suppress multiplication of the organisms,[94] which may explain the need for a somewhat prolonged 14-day course of these bacteriostatic antibiotics.

Ongoing research seeks to find:

- treatments that are safer, especially for children and pregnant women;
- therapeutic agents more effective in preventing relapse; and
- alternative treatments for antibiotic-resistant strains.[97]

Postevent Countermeasures: Current Options

Treatment. According to the consensus-based recommendations developed by the Working Group on Civilian Biodefense, the first-line therapy in a contained casualty situation (in which a modest number of individuals require treatment) is 10 days of parenteral streptomycin, with gentamicin as an acceptable substitute, for nonpregnant adults and children. Alternatives include 15 to 21 days of doxycycline or chloramphenicol or 10 to 14 days of ciprofloxacin, although treatment with tetracyclines or chloramphenicol may be more likely to result in treatment failure or relapse. For pregnant women, gentamicin (for 10 days) is preferred over streptomycin; if aminoglycosides cannot be used, alternatives include doxycycline (14–21 days) or ciprofloxacin (10 days) if the risks of their use during pregnancy are outweighed by the benefits of treating tularemia.[67]

In a mass casualty setting, in which logistics and supply limitations may preclude the use of parenteral antibiotics, treatment recommendations are identical to those described below for PEP. Treatment should begin immediately after symptom onset and continue for at least 14 days. The choice of antimicrobial should be modified based on the results of strain susceptibility testing and clinical response. Antibiotics initially administered by the intravenous route may be administered orally once the patient's condition improves.[14,66,67,70,95]

Postexposure Prophylaxis. PEP for asymptomatic individuals who have been exposed to *F tularensis* is most effective when initiated within 24 hours of exposure and continued for at least 14 days. First-line antibiotics for PEP, for adults (including pregnant women) and children, are oral doxycycline or ciprofloxacin (see Table 27-2).[67,70,95] Doxycycline and ciprofloxacin both have the potential to cause adverse effects in the fetus and young child. For an asymptomatic potentially exposed pregnant woman or child, the risk of disease must be weighed against the potential toxicity of the antibiotics. A patient at low risk of exposure could be instructed to closely monitor body temperature for 14 days, with treatment initiated if symptoms appear.[66,67,96]

Plague

Plague is an acute bacterial disease caused by *Yersinia pestis*, a nonmotile, gram-negative bacillus. Naturally occurring disease in humans is generally acquired when the bites of infected fleas result in lymphatic and blood infections (bubonic and septicemic plague, respectively). Pneumonic plague, the most deadly form of the disease, may be acquired by inhaling droplets emitted from an infected person, inhaling aerosols from infected animal tissues, inhaling *Y pestis* as an aerosolized weapon, or as a result of secondary hematogenous seeding from bubonic or septicemic plague.[100,101] Given the high mortality and person-to-person transmissibility associated with pneumonic plague, *Y pestis* is a candidate for use as a biological warfare or terrorism agent to cause pneumonic plague.

Vaccination

Formalin-Killed Plague Vaccine. The US-licensed formalin-killed whole bacillus vaccine (Greer Laboratories, Inc, Lenoir, NC) for preventing bubonic plague was discontinued in 1999. Although this vaccine and other formalin-killed plague vaccines demonstrated efficacy in the prevention or amelioration of bubonic plague based on retrospective, indirect evidence in vaccinated military troops, evidence did not support its efficacy in preventing pneumonic plague.[101–109] The vaccine's efficacy against aerosolized plague was demonstrated to be poor in animal models, and several individuals developed pneumonic plague despite vaccination.[103–109] Furthermore, these vaccines caused significant adverse reactions and required frequent boosting.[101]

Other Vaccines. A live attenuated vaccine made from an avirulent strain of *Y pestis* (the EV76 strain) has been available since 1908. This vaccine offers protection against both bubonic and pneumonic plague in animal models, but it is not fully avirulent and has resulted in disease in mice.[104] EV76 has been licensed for human use in the former Soviet Union and China for decades and has apparently caused no vaccine-related deaths, though adverse reactions are significant.[110] For safety reasons, EV76 is not used for humans in most countries.

Vaccine Research. Because of safety issues with live vaccines, recent efforts have focused on the development of a subunit vaccine using virulence factors

from the surface of the plague bacterium to induce immunity.[103,111] Two virulence factors—identified as the fraction 1 (F1) capsular antigen and the virulence (V) antigen—induced immunity and provided protection against plague in animal models. At USAMRIID, a new plague vaccine was developed by fusing the F1 capsular antigen with the V antigen to produce the recombinant F1-V vaccine. (A similar recombinant subunit vaccine formulation mixes the two antigens [F1+V].) In mice and rabbits, evidence indicates that the F1-V vaccine candidate is protective against both pneumonic and bubonic plague. In NHPs, it provided better protection than either the F1 antigen or the V antigen alone during aerosol challenge experiments.[111–113] Ongoing approaches for improving F1-V–type vaccine candidates include genetic modification of antigens and the use of different adjuvants. Phase 1 and 2 clinical trials exploring subunit plague vaccines have been recently completed. Other researchers are exploring the use of bacterial, viral, and plant live carrier platforms.[111]

Antibiotic Agents. In general, studies are lacking on the relative effectiveness of various antibiotics in the treatment or PEP of pneumonic plague in humans. Streptomycin has traditionally been the preferred treatment for plague and has been effective when initiated promptly. Gentamicin, which is more widely available than streptomycin, has also been used successfully but is not currently FDA approved to treat plague.[100] In particular, a randomized clinical trial conducted in Tanzania found that both gentamicin and doxycycline were highly effective in the treatment of all forms of plague.[114] In murine models of pneumonic plague, doxycycline and tetracycline have not consistently performed as well as other antibiotics.[100,115,116] However, the weight of experimental and anecdotal evidence for the effectiveness of doxycycline led the Working Group on Civilian Biodefense to recommend the use of the tetracycline class of antibiotics to treat plague when aminoglycosides cannot be used.[100] Fluoroquinolones also have been used successfully to treat severe cases of plague.[100] Recently, the FDA approved the fluoroquinolones levofloxacin and moxifloxacin for the treatment (and PEP) of plague based on studies demonstrating the efficacy of these antibiotics in NHPs.[117,118]

PEP with ciprofloxacin for 5 days was highly effective in mice when initiated within 24 hours after aerosol exposure to *Y pestis*, but not when initiated 48 hours after exposure.[115] Doxycycline was relatively ineffective as prophylaxis in a mouse model, even if initiated within 24 hours after aerosol exposure with mean inhibitory concentrations (MICs) ranging from 1 to 4 mg/L.[115,116] The effectiveness of doxycycline, a bacteriostatic drug, generally requires antibiotic levels to be four times the MIC. Two lines of evidence suggest that the treatment failure may be related, in part, to increased metabolism of doxycycline in mice. First, tetracycline has been used successfully in humans to treat or prevent pneumonic plague.[100,119,120] Second, doxycycline stabilized the bacterial loads in the spleens of mice infected with *Y pestis* strains with lower MICs (≤1 mg/L).[121]

Postevent Countermeasures: Current Options

Treatment. The prompt initiation of treatment (within 18–24 hours of symptom onset) is crucial, especially for primary pneumonic plague. According to consensus-based recommendations developed by the Working Group on Civilian Biodefense,[100] the first-line antibiotic for treatment of plague in adult men, nonpregnant women, and children in a contained casualty situation is parenteral streptomycin or gentamicin (although gentamicin is not FDA approved for this use); alternatives include doxycycline, ciprofloxacin, levofloxacin, moxifloxacin (in adults), or chloramphenicol. For pregnant women, gentamicin is the preferred choice, with doxycycline and ciprofloxacin as alternatives. Treatment should be continued for at least 10 days. Antibiotics initially administered intravenously can be administered orally pending improvement in the patient's condition.[14,66,100]

In a mass casualty setting, in which parenteral administration of antibiotics may not be feasible, oral doxycycline or ciprofloxacin are the preferred choices, as described below for PEP, except that treatment duration should be 10 days.[100]

Postexposure Prophylaxis. Asymptomatic individuals exposed to aerosolized *Y pestis*—as well as persons who have had unprotected face-to-face contact (within 2 meters) with patients with pneumonic plague or those potentially exposed to aerosolized *Y pestis*—should receive PEP, beginning as soon as possible and continuing for 7 days after the last known or suspected *Y pestis* exposure or until exposure has been excluded. Individuals with cough or fever within an area in which cases of pneumonic plague are known or suspected to be occurring should also be given PEP. The first-line antibiotics for PEP in adults (including pregnant women) and children are doxycycline or ciprofloxacin; chloramphenicol is an alternative, but this drug carries the risk of causing aplastic anemia. Moxifloxacin (in adults) or levofloxacin may also be appropriate (see Table 27-2).[14,66,100,105,115,116,122,123] Antibiotic sensitivity testing should be performed to assess for resistant strains. For an asymptomatic potentially exposed pregnant woman or child, the risk of disease must be weighed against the potential toxicity of the antibiotics.[66,100]

Glanders and Melioidosis

Glanders and melioidosis are zoonotic diseases caused by the gram-negative bacteria, *Burkholderia mallei* and *B pseudomallei*, respectively.[124–126] Equids serve as the natural reservoir for *B mallei*, which is generally restricted to parts of the Middle East, Asia, and South America.[127] Glanders in humans is not common and has typically been associated with contact with equids or laboratory exposure. The mode of acquisition is believed to be primarily from inoculation with infectious secretions of the animal through broken skin or the nasal mucosa and less commonly from inhalation.[11,14,66,125,128]

B pseudomallei is a natural saprophyte that can be isolated from soil, stagnant waters, rice paddies, and market produce primarily in endemic areas, such as Southeast Asia and northern Australia. However, it has been found in many tropical and subtropical regions.[124,129,130] Infection in humans is generally acquired through soil contamination of skin abrasions, but it may also be acquired by ingesting or inhaling the organism. Although symptoms of *B pseudomallei* infection are variable, the pulmonary form of melioidosis is the most common and may occur as a primary pneumonia or from secondary hematogenous seeding.[14,66,124,129]

Both *B mallei* and *B pseudomallei* have been studied in the past as potential biowarfare agents. The recent increase in biodefense concerns has renewed research interest in these organisms because of their potentially high mortality, availability, resistance to many antibiotics, and inhalational infectivity.[130–132]

Vaccination

No vaccines are available for preventing glanders or melioidosis in humans or animals. Efforts to develop vaccines are made more challenging by the propensity for both of these pathogens to develop into chronic or recurring disease.[130,131] Among the more promising lines of research are vaccines using live attenuated bacteria (which are more immunogenic in animal models) and recombinant subunit vaccines (which are less immunogenic but appear to be safer).[130,131]

Antibiotic Agents

No FDA-approved therapy or PEP exists for glanders or melioidosis. Treatment and PEP are complicated by the tendency for many strains of both *B mallei* and *B pseudomallei* to be resistant to a variety of antibiotics.[133–135] For glanders, effective treatment and PEP strategies are especially uncertain because of the rarity of the disease in humans.

Most strains of both *B mallei* and *B pseudomallei* have generally been susceptible to ceftazidime, meropenem, imipenem, ciprofloxacin, and tetracyclines. *B mallei* is also generally sensitive to rifampin and aminoglycosides, to which most isolates of *B pseudomallei* are resistant.[133–135] Resistance of *B pseudomallei* to TMP-SMZ is rare in Australia[135]; in Thailand, however, the percentage of *B pseudomallei* isolates that are resistant to TMP-SMZ may be increasing.[136]

Because of the potential for latent or recurrent *B pseudomallei* infection, which can occur several decades after exposure, treatment of melioidosis is biphasic. The first phase consists of short-term, intensive, parenterally administered antibiotics; in the second phase, antibiotics are administered orally as long-term eradication therapy.[134,137] In human cases of melioidosis, intravenous ceftazidime—with or without TMP-SMZ—has been effective during the initial intensive phase of treatment. For example, a randomized trial found a significant reduction in mortality among patients with severe melioidosis who were treated during the intensive phase with intravenous ceftazidime alone compared with those who received the conventional treatment of the time—a combination of chloramphenicol, doxycycline, and TMP-SMZ.[138] Amoxicillin/clavulanic acid (or co-amoxiclav), imipenem, meropenem, and cefoperazone–sulbactam have also been effective.[134] In particular, Cheng et al[139] found that patients with severe melioidosis who were treated with meropenem during the intensive phase had outcomes similar to those treated with ceftazidime. However, imipenem has been associated with a higher frequency of CNS adverse effects and problems for patients with impaired renal function, and co-amoxiclav may be more likely than ceftazidime to result in treatment failure.[140]

In the eradication phase of melioidosis treatment, a combination of TMP-SMZ plus doxycycline has been used successfully, as has co-amoxiclav alone.[134,141] However, a recent trial comparing combination antibiotic regimens during the eradication phase found that TMP-SMZ alone was noninferior to TMP-SMZ plus doxycycline, a combination that has been commonly recommended in Thailand. Excluding doxycycline may reduce adverse reactions and improve adherence by patients.[141] An adequate duration of the eradication phase of treatment is crucial for preventing relapse. Limmathurotsakul et al[142] have found that the most significant risk factors for relapse are choice and duration of oral antimicrobial therapy. Among patients treated during the eradication phase with an appropriate oral antibiotic regimen, such as TMP-SMZ plus doxycycline, a 12- to 16-week treatment duration reduced the risk of relapse by 90% compared with a treatment lasting no longer than 8 weeks.

Treatment of patients with glanders is based largely on experience with treating melioidosis as well as the results of animal studies. In addition to antibiotic treatment, surgical drainage of abscesses may be required for some patients.[128,134]

Most evidence on the efficacy of antibiotics as PEP for melioidosis comes from laboratory exposures. For example, among 17 laboratory workers who manipulated cultures of *B pseudomallei*, 13 individuals experienced high-risk exposure to *B pseudomallei* from sniffing culture plates and/or performing routine laboratory procedures, such as subculturing and inoculation of the organism outside a biosafety cabinet (before the organism was identified). Beginning 0 to 4 days after the exposure, 16 of the exposed workers were treated with a 3-week course of TMP-SMZ, and 1 was treated with a 3-week course of doxycycline. None of the 17 individuals developed symptoms consistent with melioidosis for 5 months after exposure.[133] However, this response may reflect the low risk of laboratory-acquired illness from the organism as opposed to the effectiveness of antibiotic prophylaxis.[143]

Animal studies are also informative for the use of antibiotics as PEP for both *B pseudomallei* and *B mallei*. In mice, TMP-SMZ initiated 6 hours after exposure to aerosolized *B pseudomallei* or *B mallei* effectively prevented acute melioidosis and acute glanders. However, these mice nevertheless succumbed to melioidosis or glanders after relapse or immunosuppression, indicating that chronic infections had been established. In rats, PEP with 10 days of quinolones or TMP-SMZ, initiated within 3 hours of subcutaneous exposure to 105 organisms of *B pseudomallei*, was completely effective in preventing disease (verified by necropsy after animals were sacrificed at 2 months postexposure).[144]

Administration of either doxycycline or ciprofloxacin (twice daily for 5 or 10 days) protected mice from disease if started 48 hours before or immediately after intraperitoneal challenge with *B pseudomallei*, though relapses occurred in a few animals within 5 weeks of discontinuation of the antibiotics. However, when the initiation of antibiotic prophylaxis was delayed to 24 hours after exposure, the treatment provided minimal protection, resulting in only a delay of infection, which occurred at least 5 weeks after discontinuation of the antibiotic.[127]

Doxycycline or ciprofloxacin (twice daily for 5 days), initiated 48 hours before or immediately after intraperitoneal challenge with *B mallei*, had a protective effect in hamsters. But the effect was temporary in some animals, with disease occurring after discontinuation of the antibiotic. Relapses were associated with both ciprofloxacin and doxycycline beginning at day 18 and day 28, respectively, after challenge. Necropsies of fatalities revealed splenomegaly with splenic abscesses from which *B mallei* could be isolated; necropsies of surviving animals revealed splenomegaly with an occasional abscess. Delay of ciprofloxacin or doxycycline prophylaxis initiation to 24 hours after exposure merely delayed disease, with relapses occurring in hamsters within 4 weeks of the challenge.[127]

The differences in results among animal models may be related—in part—to differential susceptibility among species to melioidosis and glanders. In particular, hamsters are highly susceptible to infection from *B mallei*; the protective effect of chemoprophylaxis in humans may be greater.

Postevent Countermeasures: Current Options

Treatment. According to consensus recommendations developed at the 2010 US Department of Health and Human Services Burkholderia Workshop,[140] patients with suspected or confirmed glanders or melioidosis should receive intensive therapy with intravenous antibiotics for 10 to 14 days, and until the patient's condition improves, followed by prolonged oral eradication therapy for a minimum of 12 weeks to minimize the risk of relapse. The initial intravenous treatment should be extended to greater than or equal to 4 weeks for severe disease or lack of improvement. To reduce the likelihood of relapse, the duration of oral eradication therapy should depend on disease severity and the response to treatment.[140] These consensus recommendations do not provide separate guidelines for children or pregnant women for the intensive phase of treatment. However, based on 24 years of pediatric melioidosis treatment at the Royal Darwin Hospital in the Northern Territory, Australia, McLeod et al[145] similarly recommend a minimum of 14 days of intensive intravenous therapy followed by at least 12 weeks of eradication therapy for children aged 16 years or younger with disseminated disease. For pediatric patients with localized cutaneous melioidosis, McLeod et al indicate that 12 weeks of oral antibiotic therapy (without the initial intensive parenteral treatment phase) is generally sufficient.

For intensive intravenous therapy in adults and children, ceftazidime is adequate in most cases without complications. Meropenem is an acceptable alternative and may be preferable in cases with complications, such as neuromelioidosis or persistent bacteremia, or when the patient must be admitted to an intensive care unit. Patients whose condition worsens while taking ceftazidime should be switched to meropenem. TMP-SMZ (administered intravenously, orally, or via

nasogastric tube, using the dosing for eradication therapy) may be added to this regimen for patients with severe neurologic, cutaneous, bone, joint, or prostate infections.[140,145] The optimal intravenous therapy for pregnant women is not clear; however, Wuthiekanun and Peacock[146] indicate that intravenous co-amoxiclav is used to treat pregnant women (and children) in Thailand during the intensive phase of therapy.

For eradication therapy in nonpregnant adults and in children, TMP-SMZ is the first-line antibiotic. However, potential side effects include mild allergic reactions, Stevens–Johnson syndrome, bone marrow suppression, renal failure, and liver damage. In addition, TMP-SMZ may result in adverse pregnancy outcomes. For pregnant women, patients who cannot tolerate TMP-SMZ, and cases in which the organism is resistant to TMP-SMZ, co-amoxiclav (at an amoxicillin to clavulanic acid ratio of 4:1) is an alternative eradication-phase antibiotic, but it may be associated with a greater risk of relapse.[140,145] McLeod et al[145] indicate that co-amoxiclav is also used as an alternative to TMP-SMZ in pediatric patients with melioidosis in Thailand.

Postexposure Prophylaxis. Current recommendations for PEP after suspected *B mallei* or *B pseudomallei* exposure are based largely on animal studies and in vitro work. Ideally, PEP should be initiated promptly after a known or suspected exposure and continued for a total duration of 21 days. The first-line agent for adults and children is TMP-SMZ. For cases in which the organism is resistant to TMP-SMZ or the patient cannot tolerate this antibiotic, co-amoxiclav is the second-line choice. However, although these recommendations are appropriate for small-scale (eg, laboratory) exposures, it is not clear whether the provision of PEP to all individuals potentially exposed in a large exposure event would be feasible or advisable.[140]

Because of the potential for delayed-onset disease and relapse, monitoring (including serologic testing) should continue for at least 6 months after cessation of antibiotic PEP in exposed individuals; infected individuals may require lifelong monitoring following treatment.[11,14,66,147] Seroconversion may be indicative of relapse. If relapse is suspected, treatment (as described above) should be initiated. Antibiotic regimens should be adjusted based on results of sensitivity testing.[14,66]

Brucellosis

Brucellosis, a common zoonotic disease with a global distribution, is caused by infection with one of several *Brucella* spp, including *B abortus*, *B melitensis*, and *B suis*. These intracellular, nonspore-forming, gram-negative coccobacilli can cause severe disease in humans; mortality is low, but chronic, debilitating illness can result.[54,148–150] Infection is transmitted to humans by direct contact with infected animals or their carcasses, ingestion of unpasteurized milk or milk products, and via laboratory exposure.[148] Person-to-person transmission of brucellosis has been documented, but is rare.[54,151] Brucella are highly infectious by aerosol and remain one of the most common causes of laboratory-acquired exposure,[11,152] with an infective dose of only 10 to 100 organisms.[54] In untreated survivors, chronic illness can last for years. Infection with *Brucella* spp during pregnancy, if untreated, can cause spontaneous abortion or intrauterine fetal death.[14,66] *Brucella* spp are potential agents of bioterrorism because of their widespread availability, the ease with which they can be aerosolized, their stability in the environment, and their ability to induce chronic disease.[149]

Vaccination

Live vaccines licensed for use in animals have eliminated brucellosis in most domestic animal herds in the United States, but no licensed human vaccine exists. Ongoing research is evaluating the following:

- live, attenuated vaccine candidates, in some cases encapsulated within microspheres for slow release;
- subunit vaccines;
- vaccines based on recombinant proteins;
- vectored vaccines; and
- DNA vaccines.[150]

Antibiotic Agents

No approved chemoprophylaxis exists for brucellosis, whether as treatment or as PEP. Although few studies have compared monotherapy with combination therapy in treating brucellosis, the existing evidence suggests that monotherapy is more likely to result in relapse and treatment failure.[153–155] An adequate duration of therapy is also crucial to the effective treatment of brucellosis.

In a meta-analysis of randomized controlled trials comparing various treatment regimens for brucellosis, Skalsky et al[155] found that treatment consisting of doxycycline combined with rifampin was more likely to fail (generally from relapse) than was a regimen of doxycycline plus streptomycin. Triple-antibiotic therapy with doxycycline, rifampin, and an aminoglycoside was even less likely to result in treatment failure, whereas use of a quinolone plus rifampin was among the least effective of the regimens compared.

These authors concluded that the preferred treatment should consist of two or three antibiotics, including an aminoglycoside.

In a review, Franco, Mulder, and Smits[156] noted that relapse rates with the doxycycline–rifampin regimen ranged from 16% to 40% (depending—in part—on duration of treatment), whereas the relapse rates for doxycycline–streptomycin and rifampin–minocycline were 5.3% and 2%, respectively. Monotherapy has resulted in a combined treatment failure and relapse rate as high as 50%. In a more recent meta-analysis, Solís García del Pozo and Solera[153] examined clinical trials using various antimicrobial combinations in the treatment of human brucellosis. With relapse, therapeutic failure, and adverse effect rates as the primary outcome variables, they found that the doxycycline–streptomycin combination outperformed a combination of doxycycline and rifampin. For example, across 15 studies and a total of 700 patients with brucellosis, 6 to 8 weeks of treatment with doxycycline–rifampin resulted in treatment failure or relapse in 15.2% to 16.6% of patients (in trials employing this combination for only 4 weeks, treatment failure or relapse occurred in 26.5% of 83 patients). In contrast, in 11 studies evaluating more than 700 patients, doxycycline (45 days) plus streptomycin (15–21 days) resulted in treatment failure or relapse in 6.7%–7.6% of patients. Rates of serious side effects were similar (around 1%) for both of these combinations. The doxycycline–gentamicin combination appeared to be equivalent to doxycycline–streptomycin.[153]

Evidence regarding effective PEP regimens comes largely from laboratory exposures. One study reported prophylaxis using the doxycycline–rifampin combination administered to nine asymptomatic laboratory workers who seroconverted after exposure to *B abortus* serotype 1 atypical strain (a strain with low virulence).[157] These individuals subsequently developed symptoms of fever, headache, and chills that lasted a few days. In contrast, three persons who did not receive prophylaxis had symptoms of fever, headache, and chills for 2 to 3 weeks as well as anorexia, malaise, myalgia, or arthralgia lasting an additional 2 weeks. No relapses occurred in the nine persons who received antibiotic prophylaxis, which may be a result of either the low virulence of this particular strain in humans or the early administration of antibiotic prophylaxis. In another hospital laboratory incident, six laboratory workers were identified as having had a high-risk exposure to *B melitensis* because they had sniffed and manipulated cultures outside a biosafety cabinet. Five of these individuals were given PEP for 3 weeks (four individuals received doxycycline twice daily plus rifampin once daily, and one pregnant laboratory worker received TMP-SMZ twice daily). One individual declined prophylaxis and subsequently developed brucellosis (confirmed by culture). The five individuals who received PEP remained healthy and did not seroconvert.[158] In late 2007, the CDC became aware of 916 laboratory workers in 254 laboratories with potential exposure to RB51, an attenuated vaccine strain of *B abortus* used to vaccinate cattle, during a laboratory preparedness proficiency test. PEP was recommended for the 679 individuals characterized as having had high-risk exposures and was also offered to the 237 laboratory workers with low-risk exposures. No cases of brucellosis were reported, but the number of individuals who actually received PEP has not been documented.[159]

Postevent Countermeasures: Current Options

Treatment. For uncomplicated brucellosis in adults, a combination of oral doxycycline (for 6 weeks) and intramuscular streptomycin (for 2–3 weeks) is recommended as the "gold standard" treatment in the position paper that resulted from a 2006 consensus meeting (the Ioannina recommendations).[154] Parenteral gentamicin (for 7 days) is an acceptable substitute for streptomycin. Six weeks of oral doxycycline plus oral rifampin is an alternative first-line regimen because the convenience of (and therefore, presumably, better adherence to) an entirely oral therapy is likely to overcome the drawbacks of this combination.[154] The optimal treatment for pregnant women with brucellosis has not been sufficiently studied. TMP-SMZ and/or rifampin could be considered, with risks to the fetus of antimicrobial treatment balanced against the risk of spontaneous abortion resulting from the disease (and the risk of relapse in the case of monotherapy with rifampin).[154,160,161]

In adult patients with serious complications, such as neurobrucellosis or Brucella endocarditis, the optimal antibiotic combination and treatment duration are not clear. In general, however, the duration of treatment should extend to at least 3 months. The World Health Organization (WHO) recommends the addition of either TMP-SMZ or rifampin—both of which cross the blood–brain barrier—to the doxycycline–streptomycin combination for the treatment of neurobrucellosis. Because rifampin and TMP-SMZ penetrate cell membranes, the WHO also recommends the addition of one of these antibiotics to the combination therapy for Brucella endocarditis.[160,162]

The WHO[160] recommends that children aged 8 years and older receive the same antibiotics as adults for the same duration. For younger children, the

WHO indicates that satisfactory results have been achieved using a combination of TMP-SMZ for 6 weeks and parenteral streptomycin (for 3 weeks) or gentamicin (for 7–10 days). Alternatives include TMP-SMZ–rifampin for 6 weeks or rifampin with an aminoglycoside.[160] More recent guidelines[163] developed for the treatment of children in Saudi Arabia—where brucellosis is endemic—recommend 6 weeks of treatment with a combination of rifampin and TMP-SMZ or 6 weeks of rifampin and 7 days of gentamicin for children younger than 8 years old. For more severe disease in young children, these authors recommend rifampin, TMP-SMZ, and ciprofloxacin for 3 to 9 months with gentamicin added for the first 14 days. They recommend avoidance of doxycycline in young children because of the potential for dental staining. For older children (≥8 years), they recommend doxycycline and rifampin for 6 weeks or doxycycline for 6 weeks and either streptomycin (14 days) or gentamicin (7 days). For more severe disease in older children, they suggest using doxycycline–TMP-SMZ–rifampin for 3 to 9 months with gentamicin added during the first 14 days.[163]

Postexposure Prophylaxis. For asymptomatic individuals who have had a high-risk exposure (such as exposure to laboratory aerosols or biowarfare exposure) to Brucella isolates, the CDC[164] recommends a combination of doxycycline and rifampin for 3 weeks (see Table 27-2). If that combination cannot be used, TMP-SMZ could be offered.[14,66,160,164] For an asymptomatic pregnant woman with a high-risk exposure, PEP should be considered in consultation with an obstetrician, weighing the risk of disease against the potential toxicity of the antibiotics.[164]

Q Fever

Q fever is a zoonotic disease caused by a rickettsia, *Coxiella burnetii*, a gram-negative, obligately intracellular coccobaccillus with a global distribution.[165] *C burnetii* is environmentally stable and remains viable in the soil and other substrates for weeks or potentially longer.[166] Humans typically acquire *C burnetii* infection by inhaling aerosols contaminated with the organisms (generally from the excreta of infected animals).[167] Less common routes of transmission include the consumption of unpasteurized dairy products and transmission via tick bites.[168,169] Person-to-person transmission has been reported only rarely.[170,171] Cases of Q fever among US military personnel in Iraq have been linked to tick bites and helicopter-generated aerosols.[172,173] Q fever manifests in an acute form—which may be asymptomatic—as well as a rare but potentially more serious chronic form (most often presenting as endocarditis) that can occur weeks, months, or years after the initial acute infection.[14,66,165,167,174–177] Long-term sequelae—notably, chronic fatigue and cardiovascular disease—often occur after acute infection.[175]

Vaccination

C burnetii has two major antigens, known as phase I and phase II antigens. Strains in phase I have been propagated mainly in mammalian hosts, whereas strains in phase II have been adapted to yolk sacs or embryonated eggs. Although early vaccines were made from phase II egg-adapted strains, later vaccines were made from phase I strains and demonstrated protective potencies in guinea pigs 100 to 300 times greater than vaccines made from phase II strains.[178]

No FDA-approved vaccine is available for vaccination against Q fever in the United States. However, one vaccine (Q-Vax) is approved in Australia and a similar IND vaccine (NDBR 105) has been used in at-risk researchers at Fort Detrick since 1965.

Q-Vax. Currently licensed in Australia, Q-Vax (CSL Ltd, Parkville, Victoria, Australia) has been demonstrated to be safe and effective for preventing Q fever. Q-Vax is a formalin-inactivated, highly purified *C burnetii* whole-cell vaccine derived from the Henzerling strain, phase I antigenic state.[179,180] More than 4,000 abattoir workers were vaccinated subcutaneously with 0.5 mL (30 μg) of the vaccine from 1981 to 1988. In an analysis of data through August 1989, only eight vaccinated persons developed Q fever, with all infections occurring within 13 days after vaccination (before vaccine-induced immunity) versus 97 cases in unvaccinated persons (among approximately 2,200 unvaccinated individuals, but the exact number is not known).[179] In another study, among 2,555 vaccinated abattoir workers, only two cases of Q fever were diagnosed between 1985 and 1990, with both cases occurring within a few days of vaccination (before immunity developed).[181] Nearly 49,000 individuals (primarily abattoir workers and farmers) were vaccinated between 2001 and 2004 during a national Q fever vaccination campaign in Australia. Compared with Q fever notification rates in 2001 and 2002, those in 2005 and 2006 declined by more than 50% to the lowest levels on record.[182] A recent meta-analysis of four studies assessing the effectiveness of Q-Vax in a total of 4,956 subjects found that, after excluding patients who developed symptoms of Q fever within 15 days after vaccination, the vaccine's effectiveness was 100% (with those cases included, the effectiveness was 98%).[183]

The main adverse event noted with this vaccine was the risk of severe necrosis (which resulted in sterile abscesses) at the vaccine site in vaccinees with prior

exposure to Q fever.[179,184] Therefore, a skin test using 0.02 mg of the vaccine is required before vaccination. Because of the risk of vaccine-site necrosis, vaccination against Q fever is contraindicated in persons with previous exposure to *C burnetii* as denoted by a positive skin test, which is defined as either (*a*) erythema of at least 30 mm or induration of at least 20 mm at day 1 or later after the skin test or (*b*) erythema and induration of at least 5 mm on day 7 after the test. Persons with a positive skin test are considered to be naturally immune and do not require vaccination. The exclusion from vaccination of individuals with a positive skin test has eliminated sterile abscesses (Figure 27-3).[185,186]

NDBR 105 Q Fever Vaccine. The NDBR 105 (IND 610) Q fever vaccine is an inactivated, lyophilized vaccine whose preparation is similar to that of Q-Vax. The vaccine originates from chick fibroblast cultures derived from specific pathogen-free eggs infected with the phase I Henzerling strain. NDBR 105 has been effective in animal studies.[185,187,188] The vaccine also prevented further cases of Q fever in at-risk laboratory workers in the final 4 years (1965–1969) of Fort Detrick's offensive biological warfare program, compared to an average of three cases per year before the vaccine was available.[11,189] In the 45 years of the biodefense research program at USAMRIID, only one case of Q fever (mild febrile illness with serologic confirmation)—attributed to a high-dose exposure from a breach in the filter of a biosafety cabinet—has occurred among vaccinated laboratory workers. However, the vaccine may have ameliorated disease symptoms in this case.[190]

As with Q-Vax, a skin test is required before vaccination to identify persons with prior exposure to *C burnetii*. For NDBR 105, skin testing is performed by injecting 0.1 mL of skin-test antigen (a 1:1500 dilution of the vaccine with sterile water) intradermally into the forearm.

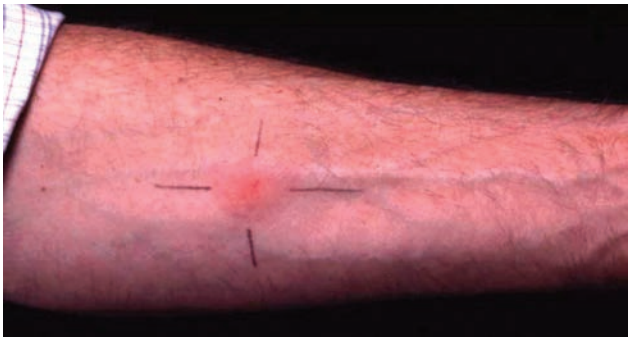


Figure 27-3. Positive Q fever skin test. Skin testing, performed by injecting 0.1 mL of skin test antigen intradermally in the forearm, is required before vaccination against Q fever to identify persons with prior exposure. Vaccination is contraindicated in individuals with a positive skin test because they are at risk for severe necrosis at the vaccine site.
Photograph: Courtesy of Herbert Thompson, MD, MPH.

The vaccine is given only once, both because it is presumed to result in lifelong immunity and because of the potential for serious local reactions in individuals with prior exposure via disease or vaccination. The vaccine is administered by injecting 0.5 mL subcutaneously in the upper outer aspect of the arm (see Table 27-1). Protection against Q fever is primarily cell-mediated immunity. Markers to determine vaccine immunity to NDBR 105 have been studied (ie, cell-mediated immunity studies, skin testing, and pre- and postimmunization antibody studies), but reliable markers have not yet been identified for NDBR 105. After vaccination with the similar Q-Vax, skin-test seroconversion occurred in only 31 of 52 persons (60%), but lymphoproliferative responses to *C burnetii* antigens persisted for at least 5 years in 85% to 95% of vaccinated persons.[179,186,191–193] Vaccine breakthroughs have been rare in vaccinated persons.

Adverse events from NDBR 105, which were reported by 72 (17%) of 420 skin-test–negative vaccinees, comprised mainly local reactions, including erythema, induration, or a sore arm. Most local reactions were classified as mild or moderate, but one person required prednisone secondary to erythema extending to the forearm. Some vaccinees experienced self-limited systemic adverse events, but these were uncommon and generally were characterized by headache, chills, malaise, fatigue, myalgia, and arthralgia.[194]

NDBR 105 is available only at USAMRIID on an investigational basis, although it is on hold (as of November 2015) because lot release data for the skin test antigen are unavailable.

Other Vaccines. Several studies are underway to explore new techniques for vaccine development, including research focusing on Th1 peptides from the major immunodominant proteins.[195,196]

Antibiotic Agents

Antibiotics are known to be effective for the treatment of Q fever, but the recommended treatment varies with the form (acute vs chronic) and severity of disease. Acute Q fever often resolves without treatment within 2 to 3 weeks.[197,198] Doxycycline, which is considered the most effective antibiotic to treat Q fever, reduces elevated body temperature within 2 to 3 days from the start of treatment; in untreated patients, fever resolves in 12.5 days (on average). Other antibiotics, including macrolides, TMP-SMZ, quinolones, and rifampin, can also be helpful, yet typically less so than doxycycline. Some

doxycycline-resistant isolates of *C burnetii* have been reported, but such resistance does not appear to be common.[198]

Among 438 patients with Q fever during an outbreak in the Netherlands, doxycycline and moxifloxacin were the first and second most commonly prescribed initial antibiotics, respectively. However, several other antibiotics were also prescribed, including potentially effective alternatives (eg, low-dose doxycycline, TMP-SMZ, ciprofloxacin, clarithromycin, and cefuroxim) as well as beta-lactam antibiotics and azithromycin, which are considered ineffective against Q fever. Patients who were treated initially with beta-lactams or azithromycin were at greatest risk of hospitalization after at least 2 days of treatment. Those receiving doxycycline at the recommended dosage (200 mg/day) had the lowest risk of hospitalization.[176]

Doxycycline has also been the most effective antibiotic to treat chronic Q fever, particularly when combined with hydroxychloroquine, which increases the bactericidal activity of the treatment. Although treatment must be continued for 18 to 24 months, the use of doxycycline alone required treatment for up to 5 years.[198] Lifelong follow-up, and sometimes lifelong treatment, may be required.[175]

Q fever infection during pregnancy, particularly during the first trimester, can result in obstetric complications as well as a greater risk of chronic Q fever for the mother, with spontaneous abortions of future pregnancies more likely.[168,175,199] Carcopino et al[199] compared maternal and fetal outcomes for 53 women who were diagnosed with Q fever during pregnancy, including 16 women who received long-term (≥5 weeks) treatment with TMP-SMZ and 37 who did not. Among the women who did not receive long-term TMP-SMZ, 81% experienced obstetric complications, including spontaneous abortion, intrauterine growth retardation, intrauterine fetal death, and premature delivery. They found that long-term TMP-SMZ during pregnancy protected against chronic Q fever in the mother, placental infection by *C burnetii*, and obstetric complications.

Postevent Countermeasures: Current Options

Treatment. According to recommendations from the CDC and the Q Fever Working Group, nonpregnant adults and older children (≥8 years old) with symptomatic acute Q fever should be treated with doxycycline. Ideally, treatment should be initiated within the first 3 days of symptom onset and continued for 14 days. Alternative antibiotics include moxifloxacin, clarithromycin, TMP-SMZ, or rifampin. Asymptomatic individuals and those whose symptoms have resolved without treatment generally should not receive antibiotic treatment, with the possible exception of individuals who are at high risk of developing chronic Q fever.[197]

For young children (<8 years old) with mild or uncomplicated illness, doxycycline should be administered for 5 days (which should not result in dental staining). If the patient remains febrile after this short course of doxycycline—or if the healthcare provider decides not to administer doxycycline at all—TMP-SMZ should be administered for 14 days.[197]

For pregnant women with acute Q fever, a longer (≥5 weeks) course of TMP-SMZ may be effective in reducing the risk of intrauterine fetal death, conversion to chronic Q fever in the mother, and adverse outcomes in future pregnancies. Treatment should not continue beyond 32 weeks' gestation because of the risk of hyperbilirubinemia. Concomitant use of folic acid may prevent antifolate effects of TMP-SMZ. However, data on the safety of Q fever treatment during pregnancy are limited; consultation with an infectious disease expert is recommended.[197]

After an acute infection, healthy patients with no risk factors for the development of chronic Q fever should be regularly evaluated for clinical and serologic signs of illness for at least 6 months after diagnosis, as described by Anderson et al.[197] Persons with risk factors for development of chronic disease should be serologically and clinically monitored more frequently and for a longer duration (at 3, 6, 12, 18, and 24 months after diagnosis of acute infection or, for pregnant women, after delivery). All patients who have recovered from an acute Q fever infection should be advised to seek immediate medical attention if symptoms of chronic Q fever reoccur at any time throughout their lives; this vigilance is particularly important for those with valvular defects or vascular abnormalities.[197]

Treatment of chronic Q fever typically involves a long course of doxycycline combined with hydroxychloroquine. A discussion of the appropriate duration of treatment, contraindications, and recommendations for the treatment of pregnant women and young children is beyond the scope of this chapter. However, these topics are discussed in depth by Anderson et al.[197]

Postexposure Prophylaxis. Limited data are available on the effectiveness of PEP for Q fever. The CDC and the Q Fever Working Group do not recommend PEP after potential exposure to *C burnetii*. Serologic and clinical (fever) monitoring is recommended for at least 3 weeks after exposure. At the first sign of fever, treatment should be initiated.[197]

VIRAL DISEASES

Vaccination is the mainstay of medical countermeasures against viral agents of bioterrorism. FDA-approved vaccines (eg, smallpox and yellow fever vaccines) and investigational vaccines (eg, vaccines against Rift Valley fever virus [RVFV] and Venezuelan, Eastern, and Western equine encephalitis viruses [VEEV, EEEV, and WEEV]) are available in the United States. Although antiviral agents and immunotherapy may be given postexposure, many of these therapies are investigational drugs with associated toxicities, and they may be in limited supply.

Encephalitic New World Alphaviruses

VEEV, WEEV, and EEEV are lipid-enveloped RNA viruses of the genus Alphavirus (family *Togaviridae*). These viruses, found in regions of North, Central, and South America, can cause severe neurologic disease in humans and equids, which typically are infected via the bite of an infected mosquito.[200,201] Infections may also be acquired via respiratory exposure to aerosolized virus, as may occur in a laboratory setting or a bioterrorism event. The VEEV complex consists of at least 13 subtypes and varieties, including epidemic/epizootic viruses, which are pathogenic to humans and equids, and enzootic viruses that are generally avirulent in equids but, in some cases, pathogenic to humans.[202–205] Humans with VEEV infections typically present with nonspecific febrile illness. However, in up to 14% of patients, VEEV causes neurologic disease.[202,203] WEEV infections typically are either asymptomatic or cause mild, nonspecific symptoms; a minority of patients experience encephalitis or encephalomyelitis. Of the three New World encephalitic alphaviruses, EEEV is the most likely to cause severe disease or death.[203] Among the survivors of encephalitis, up to 30% may experience neurologic sequelae.[14,66,202,203] Young children tend to develop more severe illness, such as encephalitis, compared with adults and older children.[200,202]

Vaccination

Vaccines are licensed for use in equids, but the only vaccines available for humans against VEE, WEE, and EEE are investigational (see Table 27-1).[201] Laboratory-acquired infections with VEEV in particular became problematic soon after discovery of the agent in 1938[206] and remain a concern.[11] Both a live attenuated VEE vaccine (TC-83) and an inactivated VEE vaccine (C-84) are available under IND status at USAMRIID. Formalin-inactivated vaccines against both EEE and WEE are also available on an IND basis at USAMRIID. These vaccines, which have demonstrated efficacy in animal models, have been used in at-risk laboratory workers at the institute for more than 50 years in the case of TC-83. However, the live attenuated vaccine, TC-83, has high reactogenicity, and the inactivated vaccines have lower immunogenicity. Also, because of their investigational status and limited supply, use of these vaccines in a bioterrorism event would be extremely limited.

Venezuelan Equine Encephalitis TC-83 Vaccine. Live attenuated VEE TC-83 vaccine (IND 142, NDBR 102) was manufactured at the National Drug Company (Swiftwater, PA) in 1965 using serial propagation of the Trinidad strain (subtype IAB) of VEEV in fetal guinea pig heart cells. The virus was plaqued once in chick embryo fibroblasts. Several VEE viral plaques were then picked and inoculated by the intracranial route into mice. The plaques that did not kill the mice were judged attenuated. One of the nonlethal plaques of VEEV was used as seed stock to propagate in the 81st passage in fetal guinea pig heart cells.[207] The TC-83 designation refers to the number of passages in cell culture. The seed stock (81-2-4), which was provided by Fort Detrick, was diluted 1:100. Five lots were produced. The bulk vaccine was stored at −80°C in 2- to 3-liter quantities at the National Drug Company (Swiftwater, PA). In 1971, the bulk was diluted 1:400 with modified Earle's medium and 0.5% human serum albumin, and then lyophilized. The freeze-dried product was then distributed under vacuum into 6-mL vials to provide convenient 10-dose vials at 0.5 mL per dose. The components of the TC-83 vaccine include 0.5% human serum albumin and 50 μg/mL each of neomycin and streptomycin. The vaccine is administered as a single 0.5 mL subcutaneous injection (approximately 10^4 plaque-forming units per dose) in the deltoid area of the arm.

Lot release testing was performed in animals, including a guinea pig safety test, mouse safety test, and guinea pig protection (potency) tests. The initial safety test challenge in the animals was a 0.5 mL dose of the vaccine (containing approximately 10^6 virions) administered intraperitoneally. All animals survived. Additional rabbit, suckling mouse, mouse virulence, and monkey neurovirulence testing were conducted. The vaccine was protective against both subcutaneous and aerosol challenge with VEEV in mice and hamsters. In a monkey model of aerosol exposure, protection was inconsistent. Periodic postrelease potency analyses have shown that infectivity for all lots has declined by one to two logs from the original data in the IND 142 submitted in 1965.[208]

At-risk laboratory workers at Fort Detrick have received the TC-83 vaccine since 1963. Administration of this vaccine to more than 6,000 individuals in initial evaluations demonstrated its excellent immunogenicity.[209] In a study of 624 vaccinees, Pittman and colleagues[210] found that 513 (82%) responded to one dose of TC-83 with an 80% plaque reduction neutralization titer ($PRNT_{80}$) of at least 1:20. However, because the vaccine is derived from epizootic strains of VEEV, it may not protect against enzootic strains and may not adequately protect against distantly related VEEV subtype IAB variants.[190]

The severity and frequency of adverse events from the VEE TC-83 vaccine vary with the vaccine lot. Among 624 vaccinees, for example, 134 (21.5%) reported self-limited reactions, primarily systemic reactions such as malaise (reported by 90 vaccinees), headache (68), fever (65), chills (50), and myalgia (43). In some vaccinees, these symptoms were severe enough to require bedrest, but in all cases symptoms resolved without permanent effects.[210] No person-to-person transmission of VEE has been documented after vaccination with TC-83. Local reactions are rarely seen.

Some evidence has hinted at an association between glucose metabolism or insulin release and either infection with VEE or inoculation with the VEE TC-83 vaccine. In most studies in humans and in animal models, results have been inconclusive or negative.[211–214] However, out of an abundance of caution, the vaccine is not given to individuals with a family history of diabetes in first-degree relatives.

The VEE TC-83 vaccine has never been evaluated in pregnant women. In 1975, one spontaneous abortion occurred as a probable complication of TC-83 vaccination. In 1985, a severe fetal malformation in a stillborn infant occurred in a woman whose pregnancy was unidentified at the time of vaccination.[215] This kind of event has been reproduced in many animal models. Rhesus monkey fetuses were inoculated with VEE vaccine virus by the direct intracerebral route at approximately gestational day 100. Congenital microcephaly, hydrocephalus, and cataracts were found in all animals and porencephaly in 67% of the cases. The virus replicated in the brain and other organs of the fetus.[216] VEE vaccine virus, which is teratogenic for NHPs, must be considered a potential teratogen of humans. The wild type VEE virus is known to cause fetal malformations, abortions, and stillbirths.[217]

Venezuelan Equine Encephalitis C-84 Vaccine. The VEE C-84 formalin-inactivated vaccine (IND 914, TSI-GSD 205) was developed in part because of the high rate of adverse reactions in humans vaccinated with TC-83. C-84, which is made from the TC-83 production seed, has undergone one more passage through chick embryo fibroblasts (the number 84 refers to the number of passages). The vaccine is then inactivated with formalin, and the resultant product is freeze dried. The VEE C-84 vaccine contains neomycin and streptomycin at a concentration of 50 μg/mL, sodium bisulfite, chicken eggs, and formalin.

In animal models, the VEE C-84 vaccine's efficacy, particularly in protecting against aerosol challenge, has been inconsistent.[218–222] However, it has successfully been used to boost human vaccinees who have previously received the VEE TC-83 vaccine.[210] Therefore, although the C-84 vaccine is not used for primary vaccination against VEE, it has been used in at-risk laboratory workers at Fort Detrick as a booster for those individuals who have received the VEE TC-83 vaccine and have either (*a*) an inadequate initial response with a $PRNT_{80}$ of no more than 1:20, or (*b*) an adequate initial response to VEE TC-83 but $PRNT_{80}$ levels that subsequently drop below 1:20.

Adverse events tend to be minor. Among 128 individuals who received C-84 as a booster, only minor local reactions occurred in 6.3% of vaccinees.[210] From 2002 to 2006 at USAMRIID, 8% to 33% of individuals receiving C-84 as a booster through the Special Immunizations Program (SIP) reported a discernible adverse event. Most reactions were mild and self-limiting local reactions of swelling, tenderness, and erythema at the vaccine site. Systemic reactions were uncommon and consisted of headache, arthralgia, fatigue, malaise, influenza-like symptoms, and myalgia. All symptoms resolved without sequelae.

The vaccine is administered as a 0.5 mL subcutaneous injection above the triceps area. The current protocol used in the SIP allows for a maximum of four doses per year if postvaccination titers are not adequate.

Western Equine Encephalitis Vaccine. The inactivated WEE vaccine (IND 2013, TSI-GSD 210) is a lyophilized product originating from the supernatant harvested from primary chicken fibroblast cell cultures.[223] The vaccine was prepared from pathogen-free eggs infected with the attenuated CM4884 strain of WEEV. The supernatant was harvested and filtered, and the virus was inactivated with formalin. The residual formalin was neutralized by sodium bisulfite. The medium contains 50 μg each of neomycin and streptomycin and 0.25% (weight/volume) of human serum albumin (US Pharmacopeia). The freeze-dried vaccine must be maintained at −25°C (±5°C) in a designated vaccine storage freezer. The National Drug Company originally manufactured the inactivated WEE vaccine. The current product, lot 3-1-92, was manufactured at the Salk Institute, Government Services Division (Swiftwater, PA) in 1992. Potency tests

have been conducted every 2 to 3 years since then, initially at the Salk Institute and then at Southern Research Institute (Frederick, MD).

Animal studies showed that the vaccine protected mice against intracerebral challenge with WEEV[224] and protected hamsters against intraperitoneal challenge.[225] The inactivated WEE vaccine protected 17 of 17 horses against intradermal challenge 12 months after vaccination, even in the absence of detectable WEE protective neutralizing antibodies.[226] Human subjects who were administered the WEE vaccine subcutaneously (either 0.5 mL at days 0 and 28 or 0.5 mL at day 0 and 0.25 mL at day 28) showed similar serologic responses. Neutralizing antibody titers did not occur until day 14 after the first dose of vaccine in each group. The mean log neutralization index was 1.7 and 1.8, respectively, at day 28 after the first dose. The antibody remained at acceptable levels through day 360 in 14 of 15 volunteers. Side effects from the vaccine were minimal, consisting primarily of headache, myalgias, malaise, and tenderness at the vaccination site.[223]

The inactivated WEE vaccine has been administered to at-risk personnel at Fort Detrick since the 1970s. Pittman and colleagues evaluated the vaccine for its immunogenicity and safety in 363 at-risk workers enrolled in evaluation trials at USAMRIID between 1987 and 1997. All volunteers received subcutaneous injections with 0.5 mL of the inactivated WEE vaccine (lot 81-1) in an initial series of three doses, administered up to day 42 (the intended schedule was 0, 7, and 28 days). For individuals whose $PRNT_{80}$ fell below 1:40, a booster dose (0.5 mL) was administered subcutaneously. Serum samples for neutralizing antibody assays were collected before vaccination and approximately 28 days after the last dose of the initial series and each booster dose. Of these vaccinees, 151 subjects (41.6%) responded with a $PRNT_{80}$ of greater than or equal to 1:40. Of 115 initial nonresponders, 76 (66%) converted to responder status after the first booster dose. A vaccination regimen of three initial doses and one booster dose provided protection lasting for 1.6 years in 50% of initial responders (unpublished data).

Passive collection was used to record local and systemic adverse events from the inactivated WEE vaccine from 1987 to 1997. Of the 363 vaccinees who received three initial injections, only 5 reported local or systemic reactions. These reactions usually occurred between 24 and 48 hours after vaccine administration. Erythema, pruritus, and induration were reported after just one of the initial vaccinations. Two volunteers also reported influenza-like symptoms after the initial dose. All reactions were self-limited. No reactions were reported after 153 booster doses.

Recent active collection of adverse events from 2002 through 2006 in the SIP revealed a reaction rate of 15% to 20% following the primary series. The reaction rate was less for booster doses than for primary series doses. The majority of these symptoms were systemic and consisted of headache, sore throat, nausea, fatigue, myalgia, low-grade fever, and malaise. The duration of these adverse events was less than 72 hours. The vaccine has not been tested for teratogenicity or abortogenicity in any animal model, nor has it been tested in pregnant women; therefore, it is not advisable to vaccinate pregnant women.

According to the current SIP protocol, the primary series of the WEE vaccine is given subcutaneously at days 0, 7, and 28; a mandatory booster is given at month 6, with subsequent booster doses (up to four in a 12-month period) administered if and when the $PRNT_{80}$ titer falls below 1:40.

Eastern Equine Encephalitis Vaccine. The Salk Institute manufactured the formalin-inactivated EEE vaccine (TSI-GSD 104) in 1989.[227] The seed for the EEE vaccine was passaged twice in adult mice, twice in guinea pigs, and nine times in embryonated eggs.[228] The final EEE vaccine was derived from supernatant fluids bearing virus accumulated from three successive passages in primary chick embryo fibroblast cell cultures prepared from pathogen-free eggs infected with the attenuated PI-6 strain of virus. The supernatant was harvested and filtered and the virus inactivated with formalin. The product was then lyophilized for storage at −20°C.

Animal studies have demonstrated that the EEE vaccine is 95% protective against intracerebral challenge with EEEV in guinea pigs, with survival correlating to serum neutralizing antibody titers.[229] Vaccination of horses was also protective against intradermal challenge at 12 months postvaccination, even with an absence of detectable neutralizing antibody titers in 16 of the 17 animals.[226]

The vaccine has been given to at-risk laboratory workers at Fort Detrick for more than 25 years. The response rate of 255 volunteers who received two primary vaccinations between 1992 and 1998 was 77.3% (197 individuals), with a response defined as a $PRNT_{80}$ of 1:40 or greater. Intradermal vaccination with the EEE vaccine resulted in an adequate titer in 66% of the initial nonresponders. Adverse events from the EEE vaccine, which occurred in approximately 20% of these individuals, consisted of headache, myalgias, and light-headedness. All symptoms subsided within several days. Mild and self-limiting local reactions of induration, erythema, pruritus, or pain at the vaccination site have also been reported (unpublished data).

The EEE vaccine contains 50 μg/mL of both neomycin and streptomycin and 0.25% (weight/volume) of human serum albumin. The initial vaccine dose is given as a 0.5 mL injection subcutaneously above the triceps area. A postvaccination $PRNT_{80}$ of 1:40 or greater is considered adequate. If the titer falls below 1:40, a booster dose of 0.1 mL should be given intradermally on the volar surface of the forearm. Booster doses must be given at least 8 weeks apart.

Vaccine Research. The live attenuated VEE vaccine candidate V3526 was scheduled to replace the 50-year-old VEE TC-83 IND vaccine. This VEE vaccine candidate, a recombinant vaccine derived from the Trinidad donkey strain of VEEV, had improved activity against VEE enzootic strains. In phase 1 clinical trials, the vaccine elicited strong immune responses. However, because of high rates of severe neurologic adverse events in these trials, further development of this product was halted. These high rates were unexpected with V3526 because it demonstrated less reactogenicity in NHP studies than the VEE TC-83 product. Recently, research in mice has suggested that a formalin-inactivated V3526 vaccine could replace C-84.[230]

Another line of research has explored the use of live chimeric Sindbis virus (an Old World alphavirus that is among the least pathogenic alphaviruses in humans) engineered to express structural proteins of VEEV, EEEV, or WEEV. Studies in animal models suggest that this approach has promise for all three New World alphaviruses. Other approaches include DNA vaccines expressing proteins of the TC-83 and Trinidad donkey strains of VEEV, viral-vectored vaccines, and nonreplicating virus-like particles.[200,201] In a recent phase 1 clinical trial, a DNA vaccine against VEEV was well tolerated, with VEEV-neutralizing antibodies detected in 100% of subjects receiving the vaccine via intramuscular electroporation and in 63% to 88% of those receiving the vaccine via intradermal electroporation.[230a]

Many of the existing New World encephalitic alphavirus vaccines have been under IND status for more than 30 years. For several reasons, including funding shortfalls, these products have never been transitioned from development to licensure.

Passive Immunotherapy

Hyperimmune serum has protected animals from lethal challenge with VEEV, WEEV, and EEEV. This line of work has progressed toward safer approaches using humanized murine monoclonal antibodies. Administration of humanized murine monoclonal antibodies against a VEEV envelope protein protected 75% to 100% of mice challenged with lethal doses of VEEV if the antibodies were given within 24 hours after exposure; delaying administration to 48 hours postexposure greatly reduced the efficacy. Similar results have been found in animal models with the administration of human antibodies or human-like (macaque) antibody fragments.[200]

Antiviral Agents

Research on antiviral compounds effective against the encephalitic New World alphaviruses remains at an early stage. Although approaches using interferons (IFNs) and toll-like receptors have shown some promise in animal models, they must be administered before and after exposure.[200] Some evidence suggests that carbodine (carbocyclic cytosine) may have potential as an antiviral agent to treat VEE postexposure.[231] Recently, Chung et al[232] reported their discovery and characterization of a novel anti-VEEV and anti-WEEV compound, the quinazolinone CID15997213. They found that this small molecule inhibited VEEV and WEEV by inhibiting viral RNA, protein, and progeny synthesis, specifically by targeting the nsP2 protein. In mice, administration of CID15997213 did not result in any signs of acute toxicity, and it provided complete protection from a lethal VEEV challenge at 50 mg/kg/day.[232]

Postevent Countermeasures: Current Options

No treatment has been shown to alter the course of VEE, WEE, or EEE in humans once disease has been contracted. At this time, treatment is limited to supportive care. No PEP exists for the New World encephalitic alphaviruses. In the context of a laboratory exposure, previously vaccinated individuals who are exposed to EEEV, WEEV, or VEEV may be offered a booster dose of the appropriate vaccine if their antibody levels are inadequate[66]; however, in a mass casualty event, limited vaccine supplies would likely preclude large-scale vaccination.

Smallpox

Smallpox is caused by variola virus, a DNA virus of the genus *Orthopoxvirus*. Once distributed globally, this disease was the greatest infectious cause of human mortality for centuries. In 1980, after an intensive vaccination program, the WHO declared that the disease was eradicated.[233] Subsequently, all known stocks of variola virus were destroyed, with the exception of stocks at two WHO collaborating centers: (1) the CDC and (2) the Russian State Research Center of Virology and Biotechnology.

Smallpox is readily transmitted from person to person via direct contact, droplets, aerosol, and contaminated fomites such as clothing and bedding.[234,235] Smallpox has been designated a category A biothreat agent because of its high mortality, high transmissibility, the potential for aerosol dissemination and transmission, and history of massive weaponization by the former Soviet Union.

Vaccination

History of Smallpox Vaccination. Vaccination against smallpox was recorded in 1,000 BCE in India and China, where individuals were inoculated with scabs or pus from smallpox victims (in either the skin or the nasal mucosa), producing disease that was milder than naturally occurring smallpox. In the 18th century in Europe, scratching and inoculation of the skin with material taken from smallpox lesions, known as variolation, was performed, resulting in a 90% reduction in mortality and long-lasting immunity. (Variolation was also performed using the pustules of a previously variolated individual.) In 1722, variolation of 242 individuals in Boston resulted in a smallpox death rate of 2.5% (6 persons) compared to a death rate of 14% in unvaccinated persons (849 deaths among 5,889 cases).[236]

In 1770, Edward Jenner noticed that milkmaids who had been exposed to cowpox virus (another orthopoxvirus) rarely had smallpox scars. Subsequently, Jenner discovered that inoculation of the skin with cowpox virus taken from a milkmaid's hand resulted in immunity. This early form of vaccination began in 1796. Beginning in the mid-1840s, the smallpox vaccine was manufactured in calfskin. The virus used as the vaccine, though originally cowpox virus, changed over time and eventually was found to be a distinct virus whose precise origins were unknown; this virus became known as vaccinia virus.[236] Production of the vaccine became regulated in 1925, with the New York City Board of Health strain of vaccinia as the primary US vaccine strain. Global vaccination efforts eventually led to eradication of the disease; the last known case of naturally occurring smallpox was reported in 1977.[233] Routine vaccination of US children ceased in 1971, and vaccination of hospital workers ceased in 1976. Finally, vaccination of military personnel was discontinued in 1989.[234,236,237]

Because of renewed concerns over the risk of bioterrorism, vaccination against smallpox in at-risk military personnel was resumed in 2003 using Dryvax (Wyeth Laboratories, Marietta, PA), a live-virus preparation of vaccinia virus (the New York City Board of Health strain) made from concentrated, lyophilized calf lymph.

Dryvax and similar first-generation smallpox vaccines, which had been used in the global smallpox eradication campaign, were known to prevent smallpox. However, Dryvax was manufactured from the lymph collected from the skin of live animals scarified with vaccinia virus. Because of risks from adventitious viruses and subpopulations of virus with undesirable virulence properties, the manufacture of a cell culture–derived (second-generation) vaccine was preferable to the animal-derived product.[238] Dryvax was replaced by ACAM2000 in 2007.[239]

Current Smallpox Vaccine. The smallpox vaccine used in the United States today, ACAM2000 (Sanofi Pasteur Biologics, Cambridge, MA), is a cell culture–based live vaccinia virus vaccine licensed by the FDA for prophylaxis against variola virus (see Table 27-1).[238] ACAM2000 is a lyophilized preparation that is free of adventitious agents and contains trace amounts of neomycin and polymyxin B. The diluent for the vaccine contains 50% glycerin and 0.25% phenol in US Pharmacopeia sterile water.[240]

Protection against smallpox is from both humoral and cell-mediated immunity; the latter provides the main protection. Humoral responses of neutralizing and hemagglutination inhibition antibodies to the vaccine appear between days 10 and 14 after primary vaccination, and within 7 days after secondary vaccination. Clinical trials have shown that administration of ACAM2000 results in cutaneous, antibody, and T cell responses that are comparable to those elicited by Dryvax. The safety profile of the two vaccines also appears to be similar.[241,242]

ACAM2000 is administered by scarification (percutaneously) to the upper arm over the deltoid muscle area with 15 jabs using a bifurcated needle.[14,240] The individual is followed after vaccination to document a take reaction, a vesiculo-papular response that indicates immunity against smallpox. Six to 8 days after the primary vaccination, a primary major reaction to the vaccine develops—a clear vesicle or pustule with a diameter of approximately 1 cm. The site then scabs over by the end of the second week, with the scab drying and separating generally by day 14 to 21 (Figure 27-4). First-time vaccinees who do not exhibit either a primary major reaction or an immune response require revaccination. If no primary reaction is noted after revaccination (and after ensuring that proper technique in vaccine administration was used), these revaccinees are considered immune.[240] At some point in the future (which may be years), the immunity of vaccinated individuals may wane, and revaccination at that time may again result in a take.

The CDC recommends vaccination with confirmation of a take at least every 10 years for laboratory researchers working with nonhighly attenuated vaccinia

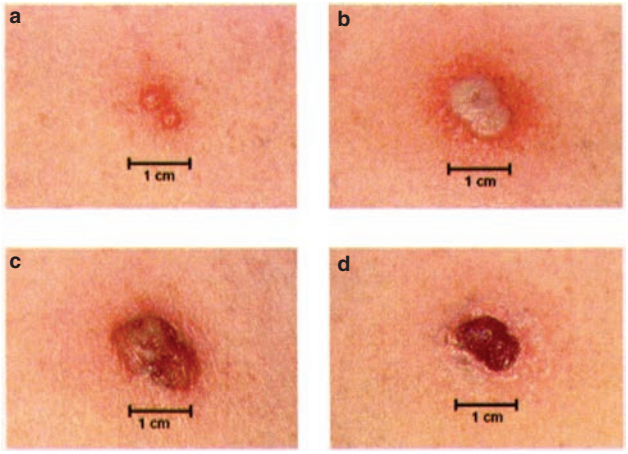


Figure 27-4. Take reaction in response to primary smallpox vaccination at (**a**) day 4, (**b**) day 7, (**c**) day 14, and (**d**) day 21. Reproduced from: Centers for Disease Control and Prevention website. http://www.bt.cdc.gov/agent/smallpox/smallpox-images/vaxsit5a.htm. Accessed September 16, 2014.

viruses, recombinant viruses developed from vaccinia viruses, and other nonvariola orthopoxviruses. For increased protection against more virulent nonvariola orthopoxviruses, such as monkeypox, revaccination every 3 years may be appropriate.[190,243] Individuals working with variola virus in the laboratory (at CDC) are required to receive a smallpox vaccination every 3 years (K S Meadows, Centers for Disease Control and Prevention, written communication, December 2015).

In the event of a smallpox release from a bioterrorism attack, individuals would be vaccinated according to the national policy. The current national policy[244] recommends vaccination initially of higher risk groups, including individuals directly exposed to the agent, household contacts or individuals with close contact to smallpox cases, and medical and emergency transport personnel. Ring vaccination—vaccination of contacts and contacts of the contacts in concentric rings around an identified active case—is the strategy that was used to control smallpox during the final years of the eradication campaign. In a postevent setting, there are no absolute contraindications to vaccination for an individual with high-risk exposure to smallpox. Persons at greatest risk of complications of vaccination are those for whom smallpox infection also poses the greatest risk. If relative contraindications exist for an exposed individual, then risks of adverse complications from vaccination must be weighed against the risk of a potentially fatal smallpox infection.

Secondary attack rates (ie, estimates of the risk of transmission from a primary case to secondary contacts of that case) from smallpox in unvaccinated persons have generally ranged from 36% to 88%, with an average rate of 58%. Household contacts in close proximity to the smallpox case for 4 hours or longer are at a higher risk for acquiring infection. In an outbreak recorded in the Shekhupura District of Pakistan during the smallpox era, the secondary attack rate was only 4% in persons vaccinated with a first-generation vaccinia virus vaccine within the previous 10 years (5/115) and 12% in persons vaccinated more than 10 years before (8/65) compared with 96% in unvaccinated persons (26/27).[245,246] Estimates of vaccine protection from imported cases of variola major between 1950 and 1971 in Western countries, where immunity from smallpox would be expected to be mainly from vaccination, showed a case fatality rate (CFR) of only 1.4% in individuals who had received the smallpox vaccine within the previous 10 years, compared with a 52% mortality rate in individuals who had never received the vaccine, 7% mortality in individuals vaccinated 11 to 20 years before, and 11% mortality in individuals vaccinated more than 20 years before. Postexposure vaccination resulted in 27% less mortality when compared (retrospectively) with smallpox patients who were never vaccinated.[245]

The effectiveness of postexposure vaccination appears to be greatest in the first 3 to 4 days after exposure to variola virus. In a recent review of historical data before the eradication of smallpox, Keckler and colleagues[247] found that vaccination of contacts decreased mortality and/or reduced morbidity in 100%, 75%, 67%, 58%, and 42% of reports when the (first-generation) smallpox vaccine was administered less than 1, 3, 5, 7, or 9 days postexposure, respectively. However, these historical data have a number of limitations, including the potential underestimation of prior immunity (from previous vaccination or exposure) in the patients described. Thus, Keckler et al also analyzed modern studies using animal models to assess the efficacy of postexposure vaccination (which, in these surrogate models, is also postinfection). In several of these studies using NHP and murine models, vaccination on postexposure day 0 or 1 resulted in 80% to 100% survival, and vaccination on postexposure day 2 or 3 resulted in 15% to 100% survival. However, in two studies, survival was consistently nearly 0% regardless of the day of vaccination. Conclusions from the animal models are difficult because of the diversity among models and the variability across species in the course of the disease.[247]

Vaccine Contraindications. According to the Advisory Committee on Immunization Practices (ACIP) and the Healthcare Infection Control Practices Advisory Committee,[248] smallpox vaccination is contraindicated in the pre-event setting for individuals who:

TABLE 27-3

CONTRAINDICATIONS TO AND PRECAUTIONS FOR PRE-EVENT SMALLPOX VACCINATION

Condition	Contraindication or Precaution
Allergy	Do not administer to those with allergies to vaccine components (eg, neomycin, polymyxin B). Where risk is great, vaccine should be administered with subsequent use of antihistamine or other appropriate medication.
Eczema (atopic dermatitis) or Darier disease (keratosis follicularis)	Do not administer to those with a history of eczema or Darier disease, even if no rash is present. Recent vaccinees should be counseled to avoid contact with individuals who have eczema or Darier disease.
Other skin conditions	Do not administer to those with disruptive or eruptive skin conditions, such as: • Severe acne • Burns • Impetigo • Contact dermatitis or psoriasis • Chicken pox The vaccine may be administered after the condition resolves or if the (noneczema/atopic) skin condition is sufficiently small and the patient is counseled to take great care to prevent transfer of vaccinia virus from vaccination site to affected skin. Vaccinees should be counseled to avoid contact with individuals who have a disruptive or eruptive skin condition.
Pregnancy or breastfeeding	Do not administer to patients who are pregnant or breastfeeding. Advise vaccinees not to become pregnant for ≥1 month after vaccination. Recent vaccinees should be counseled to avoid contact with individuals who are pregnant or breastfeeding.
Infancy	Do not administer to patients younger than 1 year old. Recent vaccinees should be counseled to avoid contact with infants.
Immunodeficiency	Do not administer to patients with diseases that have an immunodeficiency component, such as: • Human immunodeficiency virus infection • AIDS • Many cancers • Autoimmune diseases
Immunosuppressive therapy	Do not administer to patients who are currently taking immunosuppressive therapies, such as: • Cancer treatments • Some treatments for autoimmune diseases • Organ transplant maintenance • Steroid therapy (equivalent to 2 mg/kg or greater of prednisone daily or 20 mg/day if given for 14+ days), including medication for treatment of inflammatory eye disease Immunosuppression from some medications may last for up to 3 months after discontinuation.
Cardiovascular disease or risk factors	Do not administer vaccine to patients with a history of, or significant risk factors for, ischemic heart disease, myocarditis, or pericarditis or those with significant cardiac risk factors (eg, hypertension, high cholesterol, or diabetes).
Simultaneous administration of varicella vaccine	Do not administer these vaccines simultaneously because the resulting skin lesions are difficult to distinguish.
Moderate or severe illness	Do not administer vaccine to patients who are moderately or severely ill at the time of vaccination.

(**Table 27-3** *continues*)

Table 27-3 continued

Active eye disease of the conjunctiva or cornea	Patients with inflammatory eye diseases may be at increased risk for autoinoculation of the eye by touching or rubbing the eye after touching the vaccination site. Such patients can be vaccinated but should be counseled to take great care to prevent transfer of vaccinia virus to the eye.

Notes: During an outbreak, after a biological terrorism event, or for individuals with a high risk of exposure to variola virus, no contraindications are absolute. Persons at greatest risk of complications from vaccination are those for whom smallpox infection poses the greatest risk. Data sources: (1) Dembek Z, ed. *USAMRIID's Medical Management of Biological Casualties Handbook*. 7th ed. Fort Detrick, MD: US Army Medical Research Institute of Infectious Diseases; 2011. (2) Wharton M, Strikas RA, Harpaz R, et al. Recommendations for using smallpox vaccine in a pre-event vaccination program: supplemental recommendations of the Advisory Committee on Immunization Practices (ACIP) and the Healthcare Infection Control Practices Advisory Committee (HICPAC). *MMWR Recomm Rep*. 2003;52(RR-7):1–16.

- have a history or presence of atopic dermatitis (eczema);
- have active acute, chronic, or exfoliative skin conditions disruptive of the epidermis or have Darier disease (keratosis follicularis);
- are pregnant or breastfeeding;
- have conditions associated with immunosuppression;
- have a serious allergy to any of the vaccine components;
- are younger than 1 year old; or
- have close physical contact with a person who (*a*) has a history or presence of atopic dermatitis or other acute, chronic, or exfoliative skin conditions; (*b*) has a condition associated with immunosuppression; or (*c*) is pregnant (Table 27-3).[248]

The CDC has recently added underlying cardiac disease (eg, a history of ischemic heart disease, myocarditis, or pericarditis) or significant cardiac risk factors (eg, hypertension, high cholesterol, or diabetes) as relative contraindications to the vaccine in a pre-event setting; however, these exclusions may be temporary, pending the results of further research into the possible link between smallpox vaccination and cardiac disease.[249]

In addition to the contraindications listed above, the ACIP does not recommend vaccination of persons younger than 18 years old in the pre-event setting.[248] Furthermore, although the presence of an infant in the household is not an absolute contraindication for vaccination of an adult, the ACIP recognizes that vaccination programs should defer vaccination of individuals whose households include infants younger than 1 year old because of data indicating a higher risk for adverse events among primary vaccinees in this age group. Because skin lesions resulting from the varicella vaccine may be confused with vaccinia lesions, simultaneous administration of the smallpox and varicella vaccines is not recommended.[248]

During an outbreak or after an intentional release of variola virus, there are no absolute contraindications to vaccination for any person who has been exposed to smallpox. However, if pregnant or eczematous persons are vaccinated under such circumstances, vaccinia immune globulin (VIG) could be administered concomitantly.[234]

Complications of Vaccination. Vaccinia virus can be transmitted (shed) from a vaccinee's unhealed vaccination site—or from lesions caused by autoinoculation, generalized vaccinia, eczema vaccinatum, or progressive vaccinia (see below)—to other persons by close contact. The virus can survive on fomites for at least several days.[250] Contact transmission can lead to adverse events that are identical to those that could be caused by intentional vaccination. In addition, viral shedding from the vaccination site can cause autoinoculation, in which the vaccinee spreads infection from the vaccination site to other areas, such as the eye, where vaccinia virus infection is associated with significant morbidity (Figures 27-5 and 27-6).

Although medical personnel are currently taught that vaccinia virus is shed from the vaccination site only until the scab (from the take reaction) separates,[240,251] Pittman et al[252] recently found that up to 23% of vaccinees continued to shed vaccinia virus after scab separation and as late as postvaccination day 42. From December 2002 to March 2011, a period when approximately 2.1 million military personnel and 40,000 civilian emergency responders were vaccinated against smallpox with Dryvax or ACAM2000, the incidence of vaccinia transmission through contact was 5.4 per 100,000 vaccinees. Generally, the virus was transmitted to household members, intimate contacts, or sports contacts. Most cases were mild; only 1 of 115 cases was life threatening.[253] Between March 2008 and August 2010 (when only ACAM2000 was used) another group reported an incidence of contact transmission of 4.4 per 100,000 vaccinations and an incidence of autoinoculation of up to 20.6 per 100,000 vaccinations (Table 27-4).[254] To avoid inadvertent transmission, vaccinees



Figure 27-5. Accidental autoinoculation. This 22-month-old child presented after having autoinoculated his lips and cheek 9 days postvaccination. Autoinoculation involves the spread of the vaccinia virus to another part of the vaccinee's body, caused by touching the vaccination site and then touching another part of the body. Image 4655.
Reproduced from: Centers for Disease Control and Prevention Public Health Image Library website, http://phil.CDC.gov. Accessed September 16, 2014. Photograph: Courtesy of Allen W Mathies, MD, and John Leedom, MD, California Emergency Preparedness Office, Immunization Branch.

should wash their hands with soap and water or use antiseptic hand rubs immediately after touching the vaccination site and after dressing changes. Vaccinia-contaminated dressings should be placed in sealed plastic bags and disposed of in household trash. Two recent studies have demonstrated that the application of povidone iodine ointment to the vaccination site can reduce viral shedding.[252,255]

Smallpox vaccine adverse reactions are diagnosed by clinical exam. Most reactions can be managed with observation and supportive measures. Self-limited reactions include fever, headache, fatigue, myalgia, chills, local skin reactions, nonspecific rashes, erythema multiforme, lymphadenopathy, and pain at the vaccination site. Adverse reactions that require further evaluation and possible therapeutic intervention include inadvertent inoculation involving the eye, generalized vaccinia, eczema vaccinatum, progressive vaccinia, postvaccinial central nervous system disease, and fetal vaccinia (Tables 27-4 and 27-5).[256,257]

Inadvertent inoculation generally results in a condition that is self-limited unless the inoculation involves the eye or eyelid, which requires evaluation by an ophthalmologist (see Figure 27-6).[258]

Generalized vaccinia is characterized by a disseminated maculopapular or vesicular rash, frequently on an erythematous base and typically occurring 6 to 9 days after primary vaccination (Figure 27-7). Generalized vaccinia must be distinguished from other postvaccination exanthems, such as erythema multiforme and roseola vaccinatum (Figure 27-8). Lane et al reported 242.5 cases per million primary vaccinations and 9.0 cases per million revaccinations in a 1968 ten-state survey of smallpox vaccination complications.[259] The rash usually resolves without therapy. Contact precautions should be used to prevent further transmission and nosocomial infection.[258]

Eczema vaccinatum may occur in individuals with a history of atopic dermatitis, regardless of current disease activity, and it can be a papular, vesicular, or



Figure 27-6. Ocular vaccinia. This 2-year-old child presented with a case of ocular vaccinia from autoinoculation. Ocular vaccinia is an eye infection that can be mild to severe and can lead to a loss of vision. It usually results from touching the eye when the vaccinia virus is on the hand. Image 5219.
Reproduced from: Centers for Disease Control and Prevention Public Health Image Library website, http://phil.CDC.gov. Accessed September 16, 2014. Photograph: Courtesy of Allen W Mathies, MD, and John Leedom, MD, California Emergency Preparedness Office, Immunization Branch.

TABLE 27-4

RATES OF ADVERSE EVENTS AFTER SMALLPOX VACCINATION

Type of Event	Rate per 100,000 Vaccinations				
	Mar 2008–Jun 2013[*] (only serious AEs)	Mar 2008–Aug 2010[†]	Dec 2002–Mar 2011[‡]	Dec 2002–May 2003[§]	Historic Estimates[¥]
Autoinoculation	0.6	6.4–20.6	—	10.7	60.6
Contact transmission	0.5	3.5–4.4	5.4	4.7	0.8–2.7
Myo/pericarditis	1.9	—	—	8.2	10
Ischemic cardiac event	1.8	—	—	—	—
Eczema vaccinatum	0.1	—	—	0	0.2–3.5
Progressive vaccinia	0.1	—	—	0	0.1–0.7
Meningitis	0.5	—	—	—	—
Encephalitis	—	—	—	0.2	0.3–0.9
Death	0.1	—	—	0	0.1–0.2

Note: Dash indicates event not assessed.

[*]During this period, a total of approximately 834,465 doses of ACAM2000 were administered (approximately 832,035 to US military service members and 2,430 to US civilians). Events noted here are only those considered "serious" (those resulting in permanent disability, hospitalization or prolongation of hospitalization, life-threatening illness, or death). Reports of events were those submitted to the Vaccine Adverse Event Reporting System (VAERS) (see data source reference 4).

[†]During this period, 451,518 doses of ACAM2000 were administered (450,284 to US military service members and 1,234 to US civilians). The first number in each range includes only "suspect" and "confirmed" cases; the second number also includes "possible" cases. Reports included those submitted to VAERS as well as other sources (see data source reference 2).

[‡]During this period, approximately 2.1 million doses of smallpox vaccine (Dryvax until 2008, ACAM2000 thereafter) were administered to US military personnel and approximately 40,000 doses were administered to US civilians. Reports of contact transmission were assessed using the medical literature, VAERS, and the Defense Medical Surveillance System (see data source reference 1).

[§]During this period, approximately 450,293 doses of Dryvax were administered to military service members. Events noted here include those the authors considered "moderate or serious" (encephalitis, myopericarditis, eczema vaccinatum, progressive vaccinia, and death) as well as those they considered "mild or temporary" (inadvertent autoinoculation and contact transmission). Adverse events were collected in a variety of ways, including from VAERS (see data source reference 3).

[¥]From smallpox vaccinations in US civilians (adults and adolescents) and (for myopericarditis) Finnish military personnel. See Grabenstein and Winkenwerder (data source reference 3) and sources cited therein.

AE: adverse event

Data sources: (1) Wertheimer ER, Olive DS, Brundage JF, Clark LL. Contact transmission of vaccinia virus from smallpox vaccinees in the United States, 2003–2011. *Vaccine*. 2012;30:985–988. (2) Tack DM, Karem KL, Montgomery JR, et al. Unintentional transfer of vaccinia virus associated with smallpox vaccines ACAM2000 compared with Dryvax. *Human Vaccin Immunother*. 2013;9:1489–1496. (3) Grabenstein JD, Winkenwerder WJ Jr. US military smallpox vaccination program experience. *JAMA*. 2003;289:3278–3282. (4) McNeil MM, Cano M, Miller ER, Petersen BW, Engler RJ, Bryant-Genevier MG. Ischemic cardiac events and other adverse events following ACAM2000 smallpox vaccine in the Vaccine Adverse Event Reporting System. *Vaccine*. 2014;32:4758–4765.

pustular rash (Figure 27-9). Historically, eczema vaccinatum occurred at a rate of 14.1 and 3.0 per million primary and revaccinations, respectively[259]; however, in more recent military experience, no cases of eczema vaccinatum occurred in 450,293 smallpox vaccinations (of which 70.5% were primary vaccinations).[257] The rash may be generalized or localized with involvement anywhere on the body, especially areas of previous atopic dermatitis lesions.

Progressive vaccinia is a rare, severe, and often fatal complication of vaccination that occurs in individuals with immunodeficiency conditions. It is characterized by painless progressive necrosis at the vaccination site with or without metastases to distant sites (Figures 27-10 and 27-11). Those at highest risk include persons with congenital or acquired immunodeficiencies, HIV infection/AIDS, cancer, or autoimmune disease and those who have undergone organ transplantation or immunosuppressive therapy. Estimated rates of progressive vaccinia ranged from 1 to 3 per million vaccinees historically,[259] no cases in 450,293 US military vaccinees,[257] and no cases (that met case definition) in 38,440 US civilian vaccinees in 2003.[260]

Although rare, central nervous system disease, which includes postvaccinial encephalopathy and postvaccinial encephalomyelitis, is the most frequent cause of death related to smallpox vaccination.[259] Postvaccinial encephalopathy occurs more frequently than encephalomyelitis, typically affects infants and children younger than 2 years old, and reflects vascular damage to the central nervous system. Symptoms typically occur 6 to 10 days after vaccination and include seizures, hemiplegia, aphasia, and transient amnesia. Histopathologic findings include cerebral edema, lymphocytic meningeal

TABLE 27-5

VACCINIA IMMUNE GLOBULIN ADMINISTRATION FOR COMPLICATIONS OF SMALLPOX (VACCINIA) VACCINATION

Indicated	Not Recommended
• Inadvertent autoinoculation, extensive lesions or severe ocular vaccinia (without evidence of vaccinial keratitis) • Eczema vaccinatum • Generalized vaccinia, severe or recurrent • Progressive vaccinia	• Inadvertent autoinoculation, mild • Generalized vaccinia, mild (most cases) • Erythema multiforme • Vaccinial keratitis* • Central nervous system complications

Note: Data are not available on the efficacy of prophylactic vaccinia immune globulin (VIG) for a pregnant woman to prevent fetal vaccinia or on the efficacy of VIG as a treatment for an infant born with fetal vaccinia.

*VIG is contraindicated for persons with vaccinial keratitis as it may produce severe corneal opacities. An exception may be made in persons with additional, potentially fatal complications that may respond to VIG; in such cases, the choice may be one of life vs vision.

Data sources: (1) Rusnak J, ed. *Occupational Health Manual for Laboratory Exposures to Select (BSL-3 & BSL-4) and Other Biological Agents*. 3rd ed. Fort Detrick, MD: US Army Medical Research Institute of Infectious Diseases; 2011. (2) Cono J, Casey CG, Bell DM; Centers for Disease Control and Prevention. Smallpox vaccination and adverse reactions: guidance for clinicians. *MMWR Recomm Rep*. 2003;52(RR-4):1–28. (3) Lane JM, Ruben FL, Neff JM, Millar JD. Complications of smallpox vaccination, 1968: results of ten statewide surveys. *J Infect Dis*. 1970;122:303–309. (4) Lane JM, Ruben FL, Abrutyn E, Millar JD. Deaths attributable to smallpox vaccination, 1959 to 1966, and 1968. *JAMA*. 1970;212:441–444. (5) Sejvar JJ, Labutta RJ, Chapman LE, Grabenstein JD, Iskander J, Lane JM. Neurologic adverse events associated with smallpox vaccination in the United States, 2002–2004. *JAMA*. 2005;294:2744–2750. (6) Ruben FL, Lane JM. Ocular vaccinia: an epidemiologic analysis of 348 cases. *Arch Ophthalmol*. 1970;84:45–48. (7) Fulginiti VA, Winograd LA, Jackson M, Ellis P. Therapy of experimental vaccinal keratitis: effect of idoxuridine and VIG. *Arch Ophthalmol*. 1965;74:539–544. (8) Kempe CH. Studies on smallpox and complications of smallpox vaccination. *Pediatrics*. 1960;26:176–189. (9) Military Vaccine Agency. *Pregnancy Discovered after Smallpox Vaccination–IV-VIG*. Silver Spring, MD: Vaccine Healthcare Centers Network; 2013. Information Paper.

inflammation, ganglion degeneration, and perivascular hemorrhage. Patients with postvaccinial encephalopathy who survive can be left with cerebral impairment and hemiplegia. Postvaccinial encephalomyelitis, which generally affects individuals aged 2 years or older, is characterized by abrupt onset of fever, vomiting, malaise, and anorexia occurring approximately 11 to 15 days after vaccination.[258,261] Neff's 1963 national survey[262] detected 12 cases of postvaccinial encephalitis among 14,014 vaccinations. Symptoms progress to amnesia, confusion, disorientation, restlessness, delirium, drowsiness, and seizures. The cerebrospinal fluid has normal chemistries and cell counts. Histopathologic findings include demyelination and microglial proliferation in demyelinated areas with lymphocytic infiltration without significant edema. The cause for central nervous system disease is unknown, and no specific therapy exists. Intervention is limited to anticonvulsant therapy and intensive supportive care.[258,263,264]

Fetal vaccinia, which results from vaccinial transmission from mother to fetus, is a rare but serious complication of smallpox vaccination during or immediately before pregnancy (Figure 27-12). Fewer than 40 cases have been documented in the world's literature.[256]

Myopericarditis, although previously reported as a rare complication of vaccination using vaccinia strains other than the New York City Board of Health strain,



Figure 27-7. Generalized vaccinia. This 8-month-old infant developed a generalized vaccinia reaction after vaccination. Generalized vaccinia is a widespread rash involving sores on parts of the body away from the vaccination site, which results from vaccinia virus traveling through the bloodstream. Image 4644.

Reproduced from: Centers for Disease Control and Prevention Public Health Image Library website, http://phil.CDC.gov. Accessed September 16, 2014. Photograph: Courtesy of Allen W Mathies, MD, California Emergency Preparedness Office, Immunization Branch.



Figure 27-8. After receiving a smallpox vaccination on the small of his back, this 14-month-old child manifested a nonspecific rash in the form of extensive, roseola-like erythematous macules and patches over his entire body. Eruptions such as this one are common after vaccination; although often dramatic in appearance, they are largely benign and usually self-limited. There is no evidence of systemic or cutaneous spread of the vaccinia virus, and live virions cannot be recovered from the involved sites. Image 3318.
Reproduced from: Centers for Disease Control and Prevention Public Health Image Library website, http://phil.CDC.gov. Accessed September 16, 2014. Photograph: Courtesy of Arthur E Kaye, Centers for Disease Control and Prevention.

was not well recognized until reported during active surveillance of the 2002–2003 US Department of Defense (DoD) vaccination program (Figure 27-13).[265,266] In 2003, reports of myocarditis among vaccinees raised concerns about carditis and cardiac deaths in military personnel receiving the smallpox vaccine (which, at the time, was Dryvax). Among 450,293 vaccinees, 37 individuals (all men receiving their first smallpox vaccination) experienced myopericarditis, for a rate of 82 per million vaccinees.[257] Ischemic cardiac events, including (rarely) fatalities, have also been reported following vaccination with both Dryvax and ACAM2000. Most recently, McNeil and colleagues[267] reviewed ACAM2000 reports in VAERS for March 2008 through June 2013. During this period, when more than 830,000 vaccinations were administered to DoD personnel and an additional 2,430 were administered to civilians (such as research personnel), 169 reports of serious adverse events were submitted to VAERS, including 138 for which a diagnosis was verified. Of these reports, cardiac diagnoses were the most frequent, representing 54.4% of the reports; among cardiac events, myopericarditis (40.2% of cardiac reports), myocarditis (35.9%), and pericarditis (14.1%)



Figure 27-9. Eczema vaccinatum. This 28-year-old woman with eczema vaccinatum contracted it from her vaccinated child. She had a history of atopic dermatitis, which was inactive when her child was vaccinated. As a therapy, she was given vaccinia immune globulin, idoxuridine eye drops, and methisazone, which resulted in healed lesions, no scarring, and no lasting ocular damage. Image 4621.
Reproduced from: Centers for Disease Control and Prevention Public Health Image Library website, http://phil.CDC.gov. Accessed September 16, 2014. Photograph: Courtesy of Allen W Mathies, MD, California Emergency Preparedness Office, Immunization Branch.



Figure 27-10. Progressive vaccinia. This patient presented with progressive vaccinia after receiving a smallpox vaccination. Progressive vaccinia, though rare, is one of the most severe complications of smallpox vaccination and is almost always life threatening. Image 4592.
Reproduced from: Centers for Disease Control and Prevention Public Health Image Library website, http://phil.CDC.gov. Accessed September 16, 2014. Photograph: Courtesy of Centers for Disease Control and Prevention, California Department of Health Services.

were the most common diagnoses.[267] Although no clear association with vaccination has been found, a history of ischemic heart disease and the presence of significant cardiac risk pose relative contraindications for smallpox vaccination.[265–268] Consequently, individuals with a history of myocarditis, pericarditis, or ischemic heart disease should not be vaccinated.

Vaccine research. Because of the contraindications and adverse events associated with first- and second-generation smallpox vaccines, including ACAM2000, development of third- and fourth-generation smallpox vaccines is ongoing.

The highly attenuated modified vaccinia virus Ankara (MVA), a third-generation smallpox vaccine, was produced by 572 serial passages in chicken embryo fibroblasts, which rendered the virus unable to replicate in most mammalian cells. MVA, which was used toward the end of the worldwide smallpox eradication campaign, is immunogenic and safe for use even in immunocompromised individuals. MVA has been safely given to approximately 150,000 persons since it was first developed in the 1970s.[242,269,270] The safety and immunogenicity of recently developed versions of MVA, such as Imvamune (Bavarian Nordic, Martinsried, Germany), which is stored in the Strategic National Stockpile (SNS), are currently being assessed in clinical trials.[271,272] In completed clinical trials, Imvamune has been safe, well tolerated, and immunogenic, producing immune responses comparable to those elicited by Dryvax[273,274]; a trial comparing Imvamune to ACAM2000 is underway. Imvamune has received marketing authorization from the European Commission and Health Canada for immunization against smallpox in adults, including healthy individuals as well as those with immune deficiencies and skin disorders, such as atopic dermatitis and HIV infection.[275] The CDC has submitted a pre-EUA request to the FDA for potential use of Imvamune during a public health emergency; if granted, it would allow Imvamune to be administered to HIV-infected individuals and those with atopic dermatitis.[275]

Aventis Pasteur smallpox vaccine (APSV, or Wet-Vax) is a live, replication-competent liquid calf lymph-derived vaccinia virus vaccine that results in strong humoral and cellular immune responses[276]; APSV is also stored in the SNS. The CDC also has submitted a pre-EUA for the use of diluted APSV during a public emergency to increase the supply of smallpox vaccine.[275]

Fourth-generation vaccine candidates include subunit and DNA vaccines composed of vaccinia virus membrane and/or virion proteins or variola homologs.[239,270]



Figure 27-11. Progressive vaccinia after debridement. Image 4594.
Reproduced from: Centers for Disease Control and Prevention Public Health Image Library website, http://phil.CDC.gov. Accessed September 16, 2014. Photograph: Courtesy of Centers for Disease Control and Prevention, California Department of Health Services.

Passive Immunotherapy

VIG, which is administered intravenously, is used primarily for complications from the smallpox vaccine (Table 27-5); it does not currently play a role in smallpox prevention.[277] In particular, VIG may be recommended in severe cases of ocular vaccinia; however, it is contraindicated in individuals with vaccinial keratitis because of the risk of corneal clouding. Corneal clouding was observed in 4 of 22 persons with vaccinial keratitis who received VIG.[278] A subsequent study in rabbits showed that treatment of vaccinial keratitis with VIG was associated with both corneal scarring and persistent and larger satellite lesions compared with control animals.[279] VIG should not be withheld from a patient with keratitis if a comorbid condition exists that requires VIG administration and if the risk of the comorbid condition is greater than that of VIG-associated complications of keratitis.[258]



Figure 27-12. Fetal vaccinia. This child contracted vaccinia virus while in utero. Image 3338.
Reproduced from: Centers for Disease Control and Prevention Public Health Image Library website, http://phil.CDC.gov. Accessed September 16, 2014. Photograph: Courtesy of Arthur E Kaye, Centers for Disease Control and Prevention.

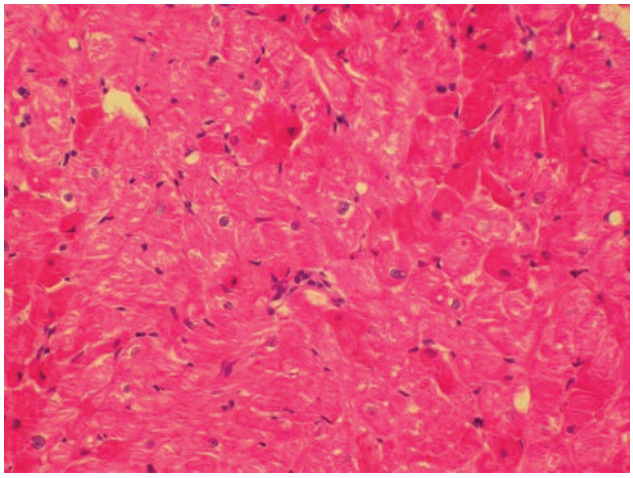


Figure 27-13. Histopathology of vaccine-related myocarditis showing a nonspecific lymphocytic infiltrate. Reproduced with permission of Department of Pathology, Brooke Army Medical Center, Texas.

Treatment of generalized vaccinia with VIG is restricted to those who are systemically ill or have an immunocompromising condition.[258]

Individuals with eczema vaccinatum are generally systemically ill and require immediate therapy with VIG, which is the only currently approved treatment for this condition.[258] The mortality rate of individuals with eczema vaccinatum was 7% (9/132), even with VIG therapy. A measurable antibody response developed in 55 of 56 survivors who had antibody titers obtained after VIG administration. No antibody response was detected in five persons with fatal eczema vaccinatum cases who had post-VIG antibody titers measured.[280]

Progressive vaccinia carries a high mortality rate and should be aggressively treated with VIG, debridement, intensive monitoring, and tertiary medical center–level support.[258] However, anecdotal experience has shown that, despite treatment with VIG, individuals with cell-mediated immunity defects have a poorer prognosis than those with humoral defects.

Prophylactic VIG to prevent fetal vaccinia could be considered for a woman who discovers she is pregnant shortly after receiving the smallpox vaccine; however, data on the efficacy of this approach are not available.[281] VIG could be considered for an infant born with lesions, but again, data regarding efficacy or the appropriate dosage are not available.[258]

Limited historical data are available on the effect of VIG in conjunction with the smallpox vaccine for postexposure prevention of smallpox in contact

cases.[282–284] A 1961 study by Kempe et al[282] demonstrated a statistically significant difference in smallpox cases among exposed contacts: smallpox occurred in 5.5% of contacts (21/379) who received the smallpox vaccine alone compared with 1.5% of contacts (5/326) who received both the smallpox vaccine and VIG therapy. Research published a year later by Marennikova[283] studied the effect of antivaccinia gamma globulin given to 13 of 42 persons who had been in close contact with smallpox patients. None of the 13 persons developed smallpox. Only 4 of the 13 individuals had a history of prior smallpox vaccination, and all but 3 of the patients were not revaccinated until day 4 after the contact. Of the 29 persons not given antivaccinia gamma globulin, 13 developed smallpox.[283] However, no clinical trials have provided evidence that giving VIG in conjunction with the smallpox vaccine as prophylaxis has a greater survival benefit than vaccination alone.[285–287]

Monoclonal antibodies represent another approach to passive immunotherapy. Postexposure administration of human monoclonal antibodies has, for example, protected rabbits from a lethal dose of an orthopoxvirus.[288]

Antiviral Agents

Antiviral agents have been used successfully, sometimes in combination with VIG, in the treatment of complications of smallpox vaccination. Animal studies suggest that some of these antivirals would also be helpful in treating smallpox infection.

Cidofovir has broad-spectrum activity against DNA viruses, including the herpes viruses, papillomavirus, adenovirus, and poxviruses.[289–291] Cidofovir provides a pronounced, long-lasting inhibition of viral DNA synthesis, allowing for infrequent (weekly or bimonthly) dosing.[292] Cidofovir has been approved by the FDA for treating cytomegalovirus retinitis in patients with AIDS. Treatment of vaccinia complications or smallpox with cidofovir would be an off-label use of the drug. However, both the DoD and the CDC currently have IND protocols for the use of cidofovir in these two conditions.

Studies of cidofovir have demonstrated improved or prolonged survival in BALB/c mice and in mice with severe combined immunodeficiency infected with vaccinia virus, as well as cowpox-infected mouse models, when treatment was initiated as long as 5 days before or up to 96 hours after infection.[293] The greatest benefit of cidofovir prophylaxis was observed when it was administered within 24 hours before or after exposure.[294–296] NHP studies have demonstrated improved survival in monkeypox and smallpox models.[297]

In humans, cidofovir has been effective in the treatment of the poxvirus infection molluscum contagiosum in patients with AIDS. Dose-related nephrotoxicity has been associated with cidofovir therapy in humans; however, this may be minimized by concomitant intravenous hydration with saline and oral probenecid (generally administered as a 2-g dose 3 hours before the cidofovir infusion, and again at 2 and 8 hours after infusion).[298–300] An investigational drug, brincidofovir (or hexadecyloxypropyl-cidofovir, previously referred to as CMX-001; Chimerix Inc, Durham, NC), is an oral formulation of cidofovir that has shown no evidence of a link to nephrotoxicity.[299] The US Government recently announced plans to add brincidofovir to the SNS for the treatment of patients with smallpox.

Tecovirimat, previously known as ST-246, is a potent and specific inhibitor of orthopoxvirus replication under development by SIGA Technologies (Corvallis, OR). The drug is active against multiple species of orthopoxviruses, including variola virus and cidofovir-resistant cowpox variants. In animal models, this oral drug has been effective in preventing death from infection with variola virus and other orthopoxviruses; it also reduced shedding of vaccinia virus after smallpox vaccination.[301,302] A recent study found that tecovirimat resulted in survival of 100% of cynomolgus macaques challenged with intravenous variola virus, whether the antiviral was administered beginning on the 2nd or 4th day after infection (only 50% of placebo-treated macaques survived). Disease in tecovirimat-treated macaques was milder, and oropharyngeal viral shedding was reduced, compared with placebo-treated survivors.[303] Clinical trials have demonstrated that tecovirimat is safe and well tolerated.[304] For treatment of orthopoxvirus infections, tecovirimat and brincidofovir may be more effective if used in combination. Furthermore, based on experience with the use of tecovirimat to treat progressive vaccinia,[305] topical tecovirimat may need to be administered instead of—or in addition to—oral tecovirimat.[304] The US Government recently added tecovirimat to the SNS for the treatment of patients with smallpox.

For the treatment of eczema vaccinatum, cidofovir can be used off-label or under an IND protocol, and the investigational antiviral drugs, tecovirimat and brincidofovir, are available under an emergency IND application.[305,306]

One animal study showed that both topical and intravenous cidofovir were effective in treating vaccinia necrosis in mice deficient in cell-mediated immunity. Topical cidofovir was more effective than intravenous cidofovir, and the administration of both

cidofovir preparations was superior to either preparation alone.[307] Again, tecovirimat and brincidofovir are available under an emergency IND application.[305,306] Because of the potential for renal toxicity with cidofovir, brincidofovir may be a better choice to treat progressive vaccinia, especially in immunocompromised individuals. It may be necessary to administer tecovirimat (particularly the topical preparation to improve absorption in individuals unable to eat) in addition to brincidofovir and repeated doses of VIG, as described by Lederman et al.[305]

The animal and human data suggest that cidofovir may be effective in therapy and in short-term prophylaxis of smallpox if initiated within 4 days after exposure. One dose of intravenous cidofovir may provide protection for 7 days.[292]

Topical treatment with trifluridine (viroptic; Catalytica Pharmaceuticals, Greenville, NC) is often recommended for ocular vaccinia resulting from inadvertent inoculation, although the FDA has not specifically approved this use of trifluridine.[258,308]

Postevent Countermeasures: Current Options

A suspected or confirmed case of human smallpox would be considered an international emergency and should be immediately reported to local and state public health authorities and the CDC. Individuals who have been exposed to smallpox patients or to animals infected with variola virus and laboratory workers with an aerosol or percutaneous exposure to variola virus must be quarantined and monitored (for fever, rash, or flu-like symptoms) for at least 17 days after the last contact with the index case or exposure, regardless of whether they have been vaccinated.[14,66]

Treatment. The FDA has not approved any antiviral agent to treat smallpox. However, tecovirimat and brincidofovir could be made available under an IND protocol to treat patients with smallpox. Tecovirimat is also available under a DoD IND and expanded access protocol for the treatment of smallpox, complications resulting from smallpox vaccination, or other orthopoxvirus infections. Intravenous cidofovir also is available to treat smallpox under an IND protocol. Clinical guidelines from the CDC for the use of antiviral medications in the event of a smallpox release or outbreak are under development.

Postexposure Prophylaxis. The CDC recently provided detailed clinical guidance on postevent use of smallpox vaccines for individuals who have been exposed to smallpox virus and those who are at high risk for smallpox infection but have not had a known exposure.[275] A brief summary of these recommendations follows.

For children and adults (including pregnant women) without severe immunodeficiency or relative contraindications who have been exposed to smallpox virus or are at high risk for infection, the CDC recommends the administration of ACAM2000. The CDC guidelines describe in detail the recommended procedure for vaccinating, in a postevent context, persons with a severe allergy to ACAM2000 or a component of that vaccine who have been exposed to the virus or are at high risk for infection. Briefly, such individuals should be vaccinated with ACAM2000, ASPV, or another vaccine, depending on the situation and the vaccines available; vaccination of such individuals may need to occur in a facility capable of treating an anaphylactic reaction. Individuals with atopic dermatitis who have been exposed to smallpox virus should be vaccinated with ACAM2000; those with atopic dermatitis who are at high risk of infection but without a known exposure should receive Imvamune instead unless they have previously received ACAM2000 without complications.

Some immunocompromised individuals—such as recipients of a solid organ transplant within the previous 3 months—are expected to benefit from vaccination with ACAM2000 (if exposed to smallpox virus) or Imvamune (if at high risk for smallpox infection without a known exposure). However, severely immunodeficient persons—such as those with HIV infection whose CD4 cell count is less than 50 cells/mm^3 or those who recently received a bone marrow transplant—are, in general, not expected to benefit from vaccination with any of the smallpox vaccines currently available. Vaccination of severely immunocompromised individuals with Imvamune may be considered if antiviral agents are not available.[275]

Viral Hemorrhagic Fevers

Viral hemorrhagic fevers (VHFs)—severe illnesses characterized by fever, vascular dysregulation, and vascular damage—are caused by a subset of the lipid-enveloped RNA viruses belonging to four families: (1) *Arenaviridae*, (2) *Bunyaviridae*, (3) *Filoviridae*, and (4) *Flaviviridae*. Transmission to humans occurs in a variety of ways, such as via infected aerosols of rodent excreta, contact with infected blood or body fluids, or through the bites of infected arthropods. Not all patients infected with these viruses develop VHF.[14,165,309,310] Because of their ability to cause widespread, severe illness and death and the potential for either aerosol dissemination and infectivity or person-to-person transmission, the viral agents of VHFs are considered potential agents of biological warfare.

Vaccination

The only vaccine for any VHF that is licensed in the United States is the live attenuated yellow fever vaccine, YF-Vax (Sanofi Pasteur Biologics, Cambridge, MA), derived from the 17D yellow fever virus strain (see Table 27-1). This vaccine has substantially diminished the burden of yellow fever infection worldwide and is well tolerated, although it is contraindicated in infants and immunosuppressed patients and is used with caution in elderly people.[311] Because of yellow fever's short (3- to 6-day) incubation period, postexposure use of this vaccine is unlikely to be effective.[309]

A number of human vaccines developed and licensed in other countries may have efficacy against VHFs. In particular, a live attenuated Argentine hemorrhagic fever (AHF) vaccine, known as Candid #1, demonstrated efficacy against Junin virus in a field study among 6,500 agricultural workers in Argentina who were randomly assigned to receive either placebo or Candid #1. Of the 23 patients who developed AHF, 22 had received placebo compared to only 1 patient who had received the vaccine.[312] Candid #1, which is the first vaccine used to control an arenaviral hemorrhagic fever, is the first live viral vaccine to be manufactured and registered in Argentina.[313]

Hantavax, a suckling mouse brain-derived hantavirus vaccine (Korea Green Cross Corporation, Yonginsi, Korea), has been licensed in South Korea since 1990. Observational trials in North Korea and China and a randomized, placebo-controlled trial in Yugoslavia supported the vaccine's efficacy[314]; however, the humoral immune response, when measured by PRNT80 antibodies, was protective in only 33.3% of vaccine recipients.[315] Clinical trials and animal studies of other hantavirus vaccine candidates, such as DNA vaccines[316,317] and vaccinia-vectored constructs,[318] have suggested other potential vaccine options.

In 1974, an inactivated Crimean–Congo hemorrhagic fever virus (CCHFV) vaccine developed by Soviet scientists was licensed in Bulgaria and is used in CCHFV-endemic areas of the country for military personnel and medical and agricultural workers. Although data are lacking on the total number of vaccinated civilians who have contracted CCHF, no vaccinated military personnel have contracted the disease since 1997, and none of the vaccinated laboratory personnel working with CCHFV have become infected, even after accidental exposure via needle prick.[319] However, a recent study found that, although CCHFV-vaccinated individuals developed high CCHFV-specific antibodies after a single dose, neutralizing activity against CCHFV was low even after repeated doses.[320]

A formalin-inactivated RVFV vaccine (TSI-GSD-200), currently under IND status, is used in the SIP at USAMRIID for laboratory workers who may be exposed to the virus (see Table 27-1).[321,322] However, no human RVFV vaccines are commercially available. The primary focus of RVFV vaccine research is the vaccination of livestock to prevent abortions and deaths in these species and spillover into humans during epizootic outbreaks.[323,324] A live attenuated vaccine for RVFV, the Smithburn strain, is the only vaccine approved for use in livestock.

Phase 1 and 2 studies have been conducted at USAMRIID on a live attenuated RVF MP12 vaccine, which was found to be safe and immunogenic in human volunteers.[325,326] Because inactivated vaccines are expensive (requiring multiple boosts) and live vaccines have side effects in animals (abortions and teratogenicity), other vaccine types are being explored for use in endemic areas. RVFV vaccines based on virus-like particles[327] or recombinant viral vectors[328] and DNA vaccines[329] have recently demonstrated potential.

A formalin-inactivated Kyasanur Forest disease virus vaccine, licensed in India since 1990, was 62.4% effective (with a 95% confidence interval of 26.1%–80.8%) and 82.9% effective (with a 95% confidence interval of 71.3%–89.8%) among those who received two doses and those who received an additional booster dose, respectively, compared with unvaccinated individuals.[330]

A neutralizing antibody study in humans and a viral challenge study in African green monkeys and crab-eating macaques demonstrated at least partial protection against two flaviviruses—Omsk hemorrhagic fever virus and tickborne encephalitis virus (TBEV)—using FSME-IMMUN, an inactivated TBEV vaccine licensed for use in Canada and Europe.[331,332]

Substantial research has focused on the development of an effective vaccine for protection against the five known antigenically distinct ebolaviruses:

1. Zaire ebolavirus (ZEBOV);
2. Sudan ebolavirus (SEBOV);
3. Taï Forest ebolavirus (also called Côte d'Ivoire ebolavirus);
4. Reston ebolavirus; and
5. Bundibugyo ebolavirus (BEBOV).[333,334]

Among the more promising approaches, several investigators have used vaccines based on viral vectors, such as recombinant vesicular stomatitis virus (VSV), that express the transmembrane glycoproteins of one or more ebolaviruses. For example, a single immunization with a replication-competent VSV vector expressing the ZEBOV glycoprotein, a vaccine candidate referred to as rVSV-ZEBOV, protected cynomolgus macaques from

lethal challenge with ZEBOV.[335,336] A single injection of a recombinant VSV vaccine expressing glycoprotein from BEBOV provided 100% protection against BEBOV challenge in macaques, as did a short prime–boost regimen using recombinant VSV–based ZEBOV and SEBOV vaccines.[337] A single immunization with a bivalent recombinant complex adenovirus vaccine (CAdVax), which expresses glycoproteins from both SEBOV and ZEBOV, protected macaques against ZEBOV challenge; with the addition of a boosting vaccination, CAdVax also provided protection against SEBOV challenge.[338] Two injections of a recombinant human parainfluenza virus type 3 vaccine vector encoding ZEBOV glycoprotein protected rhesus macaques challenged with ZEBOV.[339] Two intramuscular injections with a replicon vaccine based on SEBOV glycoprotein–expressing VEEV completely protected cynomolgus macaques challenged with aerosolized SEBOV.[340] In another approach, macaques challenged with ZEBOV were protected by three immunizations with virus-like particles containing ebolavirus glycoprotein, VP40, and nucleoprotein.[341]

Since the 2014 Ebola virus disease (EVD) outbreak in West Africa, several candidate Ebola vaccines have moved rapidly forward in development. One candidate, a recombinant chimpanzee adenovirus type 3–vectored ebolavirus Zaire vaccine (cAd3-EBOZ), recently underwent a phase 1 clinical trial in healthy adults. In this trial, a single intramuscular injection of cAd3-EBOZ was safe (2 of 10 subjects developed transient fever 1 day after vaccination) and resulted in antibody responses in the range reported to be protective in an NHP challenge model.[342] A phase 1 trial has also found rVSV-ZEBOV to be safe and immunogenic in healthy adults.[343] A phase 2/3 trial of cAd3-EBOZ and rVSV-ZEBOV is ongoing in Liberia.

Vaccines may also prove useful as PEP against some VHF-causing pathogens. In one case report,[344] a physician who experienced an accidental needlestick while working in an Ebola treatment facility in Sierra Leone during the 2014 Ebola outbreak was vaccinated 43 hours postexposure with VSV-ZEBOV through an emergency IND. Strong innate and Ebola-specific adaptive immune responses were detected after vaccination, and the patient survived.

Antiviral Agents

Antiviral medications prescribed to treat VHFs are important primarily after patients have developed symptoms because data are—in general—insufficient to support their use as PEP.

Ribavirin. The antiviral medication with the most evidence of efficacy is ribavirin, a nonimmunosuppressive nucleoside analogue with activity against a number of viruses, including at least some arenaviruses and bunyaviruses, but not filoviruses or flaviviruses.[309,345] Ribavirin inhibits the conversion of inosine 5′-phosphate (IMP) to xanthosine 5′-phosphate, disrupting the synthesis of guanosine monophosphate, a vital nucleotide needed to form viral nucleic acid.[346] However, because ribavirin does not efficiently cross the blood–brain barrier, it may not protect against neurologic effects of VHFs.[309,347] Another caveat to the use of ribavirin is its association with serious side effects, including hemolytic anemia, hypocalcemia, hypomagnesemia,[348] and genotoxicity.[349] Ribavirin has demonstrated teratogenicity and embryotoxicity in animal studies; for this reason, it is generally contraindicated during pregnancy.[309,350]

Ribavirin appears to be effective in the treatment of Lassa fever if it is begun early in the course of the illness. Among patients with Lassa fever who were treated within the first 6 days after the onset of fever, intravenous ribavirin was more effective than passive immunotherapy in reducing mortality: the CFR was reduced from 55% among patients treated with passive immunotherapy to 5% among those treated with ribavirin.[351] Results from NHP studies support this finding.[352,353] Ribavirin is less beneficial when administered starting after day 7 of illness.[345] Data are extremely limited regarding the efficacy of ribavirin PEP for Lassa fever in humans.[309,354]

Ribavirin's efficacy in treating AHF and other arenaviruses is less clear. In macaques inoculated with Junin virus on day 0, ribavirin treatment begun on day 6 (after viremia and clinical signs of illness were detected) provided minimal protection. Of the four animals, one died early in the course of illness; although initial improvement was observed in the three remaining animals, all three subsequently developed a CNS infection that was fatal in two animals.[347] However, Enria and colleagues found a survival benefit among humans with AHF who were treated with ribavirin.[355,356] An anecdotal report described recovery from Bolivian hemorrhagic fever, which is caused by Machupo virus, in two patients treated with ribavirin.[357]

In macaques, ribavirin appears to provide a benefit when used as PEP against AHF. Among macaques inoculated with Junin virus on day 0, four animals treated with ribavirin beginning on day 0 survived, whereas four that received placebo died during the 4th week after infection.[347]

In a double-blind, placebo-controlled trial, ribavirin effectively reduced mortality and viremia in patients with hemorrhagic fever with renal syndrome (HFRS).[358,359] However, a meta-analysis of the use of ribavirin in the treatment of HFRS and hantavirus pulmonary syndrome found mixed results.[360]

Ribavirin also has demonstrated in vitro activity against CCHFV.[348,361] The results of human studies assessing the efficacy of ribavirin in the treatment of CCHF are highly variable.[362–365] However, as Ergonul and colleagues argue, this variability may be due—at least in part—to variability in the delay between symptom onset and the start of treatment.[366,367] In one study, for example, patients admitted to a hospital and started on ribavirin within 2 days of symptom onset were less likely than others to become more severe cases.[367]

As PEP, evidence of ribavirin's efficacy in humans is limited. In one case study,[368] six healthcare workers who were exposed to CCHFV (via needlestick or contact with skin and mucosal surfaces) received ribavirin beginning within 1 hour of exposure; none of these individuals developed symptoms. One healthcare worker who was exposed to CCHFV (probably via aerosolization of contaminated blood or secretions) and did not receive ribavirin later developed CCHF (she recovered fully after treatment with ribavirin).[368]

Favipiravir. A viral RNA polymerase inhibitor, favipiravir (also known as T-705 and marketed as Avigan; Toyama Chemical Co, Shinjuku-ku, Tokyo) was initially developed as an antiinfluenza drug. It has been approved in Japan for specifically defined cases of influenza. However, it may also be effective against several other virus families, including arenaviruses, bunyaviruses, and flaviviruses.

During and after the 2014 EVD outbreak in West Africa, interest in favipiravir increased dramatically. A phase 2 clinical trial is underway in Guinea, aiming to assess the efficacy of favipiravir in reducing mortality in individuals with EVD. Preliminary results posted by the group Médecins sans Frontièrs suggest that 10 days of favipiravir may be beneficial among patients (children older than the age of 1 year and nonpregnant adults) with high or moderate levels of viral replication who have not yet developed severe visceral lesions. However, the drug appears not to be efficacious among those with a very high level of viral replication along with serious visceral involvement.[369] Based on the apparently greater benefit of favipiravir for patients with moderate to high viremia versus very high viremia, Van Herp et al[370] argue for the use of favipiravir as PEP for contacts of patients with EVD.

In animal models, favipiravir has also shown promise to treat AHF and CCHF. Favipiravir resulted in 78% survival of guinea pigs infected with Junin virus when administered intraperitoneally for 2 weeks beginning 2 days after challenge; by comparison, only 11% of placebo-treated animals survived. Among ribavirin-treated guinea pigs, survival ranged from 33% to 40%. Oral administration of favipiravir was less protective than was the intraperitoneal route, with 20% of orally treated guinea pigs surviving; however, animals that succumbed survived longer than placebo-treated animals.[371] In a small animal model of CCHF, mice treated with favipiravir, initiated up to 2 days after infection with CCHFV, survived with no signs of disease and no virus detectable in blood or organs.[372,373]

BCX4430. The synthetic adenosine analogue BCX4430 has broad-spectrum antiviral activity against many viruses, including bunyaviruses, arenaviruses, flaviviruses, and filoviruses. In particular, Warren et al[373] recently found that BCX4430, administered as late as 48 hours after infection, completely protected macaques from disease caused by Marburg virus. This product also conferred significant protection in guinea pigs challenged with Marburg virus and in mice challenged with Ebola virus. A phase 1 clinical trial to assess the safety, tolerability, and pharmacokinetics of BCX4430 in healthy adults was recently completed.

Interferons. Stimulating the immune system is another potential therapeutic modality, but no human studies using this technique have been conducted for any of the VHF viruses. IFN combinations may be useful in such an approach, particularly with VHF infections in which the immune response is impaired. However, IFN compounds may be deleterious in some VHF infections, such as AHF, in which high IFN levels are associated with worse outcomes.[310,374] IFNs have demonstrated a benefit in bunyavirus murine models.[375] In NHPs inoculated with Ebola virus, early postexposure treatment with either IFN α-2b[376] or IFN-β[377] prolonged survival but did not prevent death. Similar findings were obtained in Marburg virus–inoculated macaques that received early postexposure treatment with IFN-β.[377]

Other Drugs. Although in vitro data suggest that the Mx family of proteins has antiviral activity against a wide variety of RNA viruses, further study is needed.[378,379] Recently, FDA-approved IND applications and phase 1 clinical trials have been initiated for two small-molecule therapeutics: (1) anti-sense phosphorodiamidate morpholino oligomers (AVI-6002, AVI-6003) and (2) lipid nanoparticle/small interfering RNA (TKM-Ebola). However, the need for multiple doses to achieve therapeutic efficacy makes these compounds less than ideal with regard to patient compliance and outbreak scenarios.[380]

Pathogenesis studies with Ebola virus have implicated tissue factor-induced disseminated intravascular coagulation as a critical component of fatal outcomes.[381] In a rhesus macaque model of Ebola virus infection, treatment with a factor VIIa/tissue factor inhibitor (recombinant nematode anticoagulation protein c2 or rNAPc2) led to a survival advantage[382]; however, rNAPc2 was not effective against Marburg virus.[383]

This compound has not been tested in humans for treating EVD, and tissue factor inhibitors have not been effective in the treatment of septic shock.[384]

IMP dehydrogenase inhibitors (similar to ribavirin) have been tested in both in vitro and animal models against arenaviruses; however, because of their toxicity, such compounds have been used only experimentally for cancer patients in crisis.[385,386] Other compounds that have demonstrated in vitro activity against arenaviruses include 3′-fluoro-3′-deoxyadenosine,[387] phenothiazines,[388] and myristic acid compounds.[389,390] When challenged with Lassa virus, guinea pigs treated with ST-193, a small-molecule inhibitor of arenavirus entry into cells, had an overall survival rate of 62.5% compared with 0% in the ribavirin-treated and vehicle groups.[391]

Although using steroids to treat VHFs has not been recommended,[309] evidence suggests that corticosteroids may be effective among severely ill patients with CCHF. In a recent study, among 16 severely ill patients with CCHF who received corticosteroid therapy in addition to ribavirin, 8 died (a CFR of 50%), whereas among 8 severely ill patients who did not receive additional corticosteroid therapy, all 8 died (a CFR of 100%; $P = 0.014$). Among moderately ill patients, corticosteroid was not associated with a reduced CFR.[392] Several antivirals have been tested in a bunyavirus (Punta Toro virus) murine model,[375] suggesting possible compounds for further testing.

Passive Immunotherapy

Studies on the benefits of passive immunotherapy for treating VHFs have yielded mixed results.[309] Serum collected from donors after infection with Junin virus has been used successfully to treat AHF.[355,393] In a cynomolgus macaque model of Lassa virus infection, treatment with serum from immune monkeys led to a survival advantage; the benefit was greater when this passive immunotherapy was combined with ribavirin.[353] In humans, however, serum from convalescent patients used to treat Lassa fever did not reduce mortality in patients with a high risk of a fatal outcome.[351] Human-derived antibodies against Bolivian hemorrhagic fever dosed in rhesus macaques to achieve neutralizing antibody titers of 1:4 to 1:8 protected monkeys against severe clinical manifestation of illness after Machupo virus challenge.[394]

Anecdotal evidence suggests that immunoglobulins and/or transfusions from convalescent patients may improve outcomes in human EVD.[395,396] Postexposure treatment with concentrated polyclonal IgG antibodies collected from vaccinated macaques that survived an Ebola or Marburg challenge was completely protective in macaques challenged with Ebola or Marburg virus[397]; in contrast, an earlier study using equine IgG did not produce a mortality benefit in NHPs.[376] ZMapp, a product composed of three humanized monoclonal antibodies produced in the plant *Nicotiana benthamiana* completely protected macaques when treatment was initiated up to 5 days after Ebola virus challenge.[398,399] ZMapp is now undergoing a clinical trial to assess its safety and efficacy in the treatment of EVD.

Substantial supportive data are lacking for the use of immunoglobulin from survivors for treating CCHF,[319] but 15 high-risk patients (viral load of at least 108 copies/mL) treated with convalescent hyperimmunoglobulin had a survival rate of 86.6%,[400] and an earlier small case series found 100% survival among treated patients.[401] Monoclonal antibodies against HFRS viruses have been effective in murine models,[402] and such treatment appears to be well tolerated in healthy human volunteers.[403]

Yellow fever virus immunotherapy data from human studies are lacking; however, specific monoclonal antibody therapy with MAb 2C9-cIgG resulted in substantial improvement in survival among hamsters infected with yellow fever virus.[404]

As with passive immunotherapy for treating other diseases, concerns about the transmission of bloodborne pathogens, such as hepatitis C,[405] may limit treatment with donated serum or may—at a minimum—necessitate a rigorous screening process. In addition, the impracticality of obtaining large quantities of donated serum from previously infected individuals with no such population available (particularly in the United States) limits the utility of this treatment. Revolutionary advances in plant virus-based transient expression to manufacture large quantities of monoclonal antibodies may facilitate passive treatment with antibodies to counteract the effects of VHFs.[405]

Other Countermeasures

Good infection control practices, particularly the isolation of patients and barrier precautions, are a crucial countermeasure in efforts to limit the impact of VHF viruses used as biological weapons. The specific infection control needed for each virus is discussed elsewhere in this volume. Management measures also must overcome the fear and panic associated with use of a VHF virus whose potential lethality tends to be exaggerated in popular culture, such as Ebola.[406] Modern intensive care unit support with careful fluid management will probably improve the outcome for patients infected with VHF viruses,[407] but access to this care may be limited in a mass casualty scenario.

Postevent Countermeasures: Current Options

Treatment. Supportive care is the primary form of treatment of individuals with VHFs. For adults (including pregnant women) and children with a VHF of unknown etiology, the Working Group on Civilian Biodefense[309] recommends treatment with ribavirin and supportive care, beginning as soon as possible after symptom onset. In the case of a VHF, the potential teratogenic and embryotoxic effects of ribavirin are thought to be outweighed by the benefits of treatment. If the VHF is found to be caused by an arenavirus or bunyavirus, then the ribavirin should be continued such that the patient is treated for 10 days. If the infection is caused by a filovirus or flavivirus, ribavirin should be discontinued. In a contained casualty situation, ribavirin should be administered intravenously (under an IND protocol); in a mass casualty situation, it should be administered orally (an off-label use).[309] The DoD maintains expanded access protocols for the IND use of intravenous ribavirin to treat Lassa fever, CCHF, and HFRS caused by Hantaan, Seoul, Puumala, and Dobrava viruses. Patients with AHF or Bolivian hemorrhagic fever may benefit from convalescent plasma, which is used as an investigational therapy.[14]

Postexposure Prophylaxis. In the context of a bioterrorism event, the Working Group on Civilian Biodefense[309] recommends careful observation of exposed patients for 21 days, with antiviral treatment begun only if fever or other signs and symptoms of infection appear. Persons with a high-risk exposure to a VHF-causing virus and close contacts of patients with a VHF (other than RVF or a flavivirus-caused VHF, which are not transmitted person to person) should be instructed to record their temperature twice daily and report any symptom of a VHF, including a temperature of 101°F or higher. The appearance of symptoms should prompt the initiation of treatment as described previously.[309]

For asymptomatic laboratory workers or healthcare workers, a high-risk exposure (eg, via needlestick) to Lassa virus, CCHFV, or a hantavirus could warrant PEP with ribavirin[408–410]; although this recommendation is not a product of a consensus process.

TOXINS

Botulinum Neurotoxin

Clostridium botulinum is an anaerobic, gram-positive, spore-forming bacillus that produces a potent toxin, botulinum neurotoxin (BoNT). The most poisonous substance known, BoNT is found in soil and water worldwide and is commercially available for cosmetic and medical uses.[165,411] By blocking the release of acetylcholine, a neurotransmitter that causes muscle contraction, BoNT may result in muscle weakness, flaccid paralysis, and subsequent respiratory impairment. Eight immunologically distinct toxin serotypes (A through H) are produced by discrete strains of the organism. Although botulism is generally acquired from ingestion of food contaminated with BoNT, it may also occur from toxin production by *C botulinum* if present in the intestine or wounds. Botulism is not acquired naturally by aerosolization; this route of acquisition would suggest a possible bioterrorism event but may also occur from exposure to aerosolized toxin in a research laboratory.[1]

Vaccination

Pentavalent Botulinum Toxoid. No FDA-licensed vaccines are available for preexposure vaccination against botulism. An investigational product, pentavalent botulinum toxoid (PBT), was used from 1959 through 2011 for persons at risk for exposure to BoNT serotypes A through E. The PBT was available as an IND through the CDC (IND-161, for at-risk laboratory workers) until it was discontinued based on data indicating a decline in immunogenicity of some of the toxin serotypes.[412] The PBT has also been available through the US Army Office of the Surgeon General (IND-3723, for at-risk military personnel). Although IND-3723 remains active, the PBT is now effective only against toxin serotype A. Thus, the PBT could still be used to vaccinate military personnel against toxin serotype A.[413] Derived from formalin-inactivated, partially purified toxin serotypes A, B, C, D, and E, the PBT was developed by the DoD and manufactured first by Parke Davis and later (beginning in the early 1970s) by the Michigan Department of Public Health. Each of the five toxin serotypes was propagated individually in bulk culture and then underwent acid precipitation, filtration, formaldehyde inactivation, and adsorption onto an aluminum phosphate adjuvant. The five individual toxin serotypes were then blended to produce the end product[413–415]

Vaccine Research. Vaccine candidates include formalin-inactivated toxoids (A through F), which are made in nearly the same way as formalin-inactivated PBT, and recombinant BoNT vaccines.[416,417] The production of formalin-inactivated toxoids is expensive and relatively time consuming because it (*a*) requires partially purified culture supernatants to be treated exhaustively with formaldehyde and (*b*) must be

performed by a highly trained staff within a dedicated high-containment laboratory space. Furthermore, the resulting toxoid is relatively impure, containing only 10% neurotoxoid (the remainder is irrelevant material).

However, the use of pure and concentrated antigen in recombinant vaccines offers advantages—increased immunogenicity and decreased reactogenicity—over formalin-inactivated toxoids.[414] Recombinant techniques use a fragment of the toxin that is immunogenic but is not capable of blocking cholinergic neurotransmission. Both *Escherichia coli* and yeast expression systems have been used in the production of recombinant fragments, mainly the carboxy-terminal fragment of the heavy chain (Hc) of the toxin.[414,418] Phase 1 trials on the bivalent recombinant vaccine (for protection against toxin serotypes A and B) have been completed, with promising preliminary serologic results at 12 months after two doses of vaccine (administered at 0 and 6 weeks).[419] DynPort Vaccine Company LLC (Frederick, MD) sponsored a phase 2 randomized, double-blind, placebo-controlled, multicenter study to evaluate the safety, dosing schedule, and antibody kinetics of recombinant botulinum vaccine A/B (rBV A/B-40) in healthy adults. It was completed in December 2010, but results have not been published. A phase 3 randomized study to assess the safety, lot consistency, and clinical benefit of rBV A/B is planned. Recombinant vaccines given by aerosol[420,421] and by the mucosal route[422] are also being investigated.

Because the BoNT Hc has been produced as a stable recombinant protein and is an excellent immunogen, it has been assessed in diverse viral delivery platforms.[413] In particular, BoNT Hc has been virally vectored using attenuated human adenovirus,[423,424] inactivated rabies virus virions,[425] and Semliki Forest virus (SFV) DNA replicon[426,427] or recombinant SFV viral replicon particles,[428] conferring substantial protection against lethal challenge in murine models.

Passive Immunotherapy

In March 2013, the FDA approved BAT (Botulism Antitoxin Heptavalent [A, B, C, D, E, F, G] – Equine) to treat individuals with symptoms of botulism following a known or suspected exposure. BAT was developed at USAMRIID, as one of two equine-derived heptavalent BoNT antitoxins, and manufactured by Cangene Corporation (Winnipeg, MB, Canada), which is now Emergent BioSolutions (Rockville, MD). The first approval of a plasma derivative under the Animal Rule, BAT is a sterile solution of fragments of antibodies to seven of the eight BoNT serotypes known to cause botulism (A, B, C, D, E, F, and G, but not H). The antibody fragments are derived from the processing of whole antibodies obtained from horses previously immunized with a specific serotype. When administered to humans, the most commonly observed side effects include headache, fever, chills, rash, itching, and nausea. However, BAT has the potential to cause hypersensitivity reactions, including anaphylactic and anaphylactoid reactions, in individuals sensitive to equine proteins; delayed allergic reactions may occur 10 to 21 days after administration. Therefore, a skin test before administration of BAT and careful monitoring is advised. BAT is approved for use in adults and children, including infants with botulism caused by serotypes other than A or B. The safety of BAT in pregnant and lactating women is unknown; evidence regarding safety and efficacy in pediatric and geriatric populations is limited. BAT is maintained in the SNS and is available through the CDC's Drug Service.[429,430]

In October 2003, the FDA approved the Botulism Immune Globulin Intravenous (Human) (BabyBIG), a human botulism immune globulin derived from pooled plasma of adults immunized with PBT, for the treatment of infants with botulism from toxin serotypes A and B. Because the product is derived from humans, BabyBIG does not carry the high risk of anaphylaxis observed with equine antitoxin products or the risk of lifelong hypersensitivity to equine antigens. BabyBIG may be obtained from the California Infant Botulism Treatment and Prevention Program through the California Department of Health Services.[431]

Although passive antibody prophylaxis has been effective in protecting laboratory animals from toxin exposure,[432] the limited availability and short-lived protection of antitoxin preparations make preexposure or postexposure prophylaxis with these agents impractical for large numbers of people. Additionally, the administration of equine antitoxin in asymptomatic persons is not recommended because of the risk of anaphylaxis from the foreign proteins. However, if passive immunotherapy is given, it should be administered within 24 hours of a high-dose aerosol exposure to botulinum toxin.

Postevent Countermeasures: Current Options

Treatment. Immediately after clinical diagnosis of botulism, adults (including pregnant women) and children should receive a single intravenous infusion of antitoxin (BAT or, for infants with botulism from serotypes A or B, BabyBIG) to prevent further disease progression. The administration of antitoxin should not be delayed for laboratory testing to confirm the diagnosis.[14,66,430,433–435] Skin testing should be conducted

before the administration of BAT to detect sensitivity to serum or antitoxin.[430,435] Intensive supportive care (eg, artificial ventilation or feeding by enteral tube) should also be provided.[434] Although antibiotics may be necessary for the treatment of wound botulism or secondary infections, aminoglycosides and clindamycin should be avoided because they may further impair neuromuscular transmission.[434]

Postexposure Prophylaxis. Asymptomatic individuals with suspected exposure to BoNT should be carefully monitored, preferably near critical care services, for evidence of botulism; the patient's vital capacity and maximal expiratory force should be assessed frequently. Such individuals should be treated promptly with antitoxin at the first sign of illness.[434] In rare instances, it may be appropriate to administer antitoxin as PEP to asymptomatic persons after a high-risk laboratory exposure. PEP may also be appropriate for asymptomatic persons who are thought to have been exposed concurrently with persons already diagnosed with botulism.[14,66]

Staphylococcal Enterotoxin B

Staphylococcal enterotoxin B (SEB) is one of more than 20 antigenically distinct enterotoxin proteins produced by the bacterium *Staphylococcus aureus*. Ingestion of SEB is a common cause of food poisoning, with symptoms (including nausea, vomiting, and diarrhea) typically beginning within 1 to 6 hours of exposure. Ocular exposure can result in conjunctivitis and localized periocular swelling and sometimes gastrointestinal symptoms. Inhalation of SEB may cause fever, fatigue, respiratory symptoms, and sometimes gastrointestinal symptoms, generally within 2 to 12 hours of exposure, which may progress to overt pulmonary edema, acute respiratory disease syndrome, septic shock, and death.[66,436] Because it can be disseminated in a variety of ways and can cause lethal shock in humans, even at low doses (especially by the inhalational route), SEB is considered a potential bioterrorism agent.

Vaccination

No vaccine against SEB is available. However, several candidate vaccines have demonstrated protection against SEB challenge in animal models. These vaccines are based on a correlation between human antibody titers and the inhibition of T cell response to bacterial superantigens. A recombinantly attenuated SEB vaccine given by nasal or oral routes, using cholera toxin as a mucosal adjuvant, induced both systemic and mucosal antibodies and provided protection in mice against intraperitoneal and mucosal challenge with wild type SEB.[437] Intramuscular vaccination with recombinantly attenuated SEB using an Alhydrogel (Accurate Chemical & Scientific Corporation, Westbury, NY) adjuvant was protective in rhesus monkeys challenged by aerosols of lethal doses of SEB. All monkeys developed antibody titers, and the release of inflammatory cytokines was not triggered.[438] A phase 1 clinical trial assessing the safety and immunogenicity of a recombinant SEB vaccine has recently been completed.

A candidate SEB vaccine using a VEEV replicon as a vector has also been studied. The gene encoding mutagenized SEB was cloned into the VEEV replicon plasmid, and the product was then assembled into VEEV replicon particles. The vaccine elicited a strong antibody response in animal models and was protective against lethal doses of SEB.[439]

SEB toxoids (formalin-inactivated) incorporated into meningococcal proteosomes or microspheres have been found to be immunogenic and protective against aerosol SEB challenge in NHPs. The proteosome-toxoid, given intratracheally, elicited serum IgG and IgA antibody titers as well as a strong IgA response in bronchial secretions.[440] Vaccination by an intratracheal route with formalinized SEB toxoid-containing microspheres resulted in higher antibody titers in the serum and respiratory tract, a higher survival rate, and a lower illness rate than booster doses given by intramuscular or oral routes. (Microspheres provide controlled release of the toxoid, which results in both a primary and an anamnestic secondary antitoxin response and thereby may require fewer doses.)[441] However, enteric symptoms such as vomiting still occurred in many vaccinees with both vaccine candidates.[440–442]

Passive Immunotherapy, Postexposure Prophylaxis, and Treatment

No PEP is available for SEB. The only current treatment modality is intravenous human immunoglobulin. This form of passive immunotherapy can reduce mortality in animal models if given within 4 to 8 hours after inhalation.[66]

Ongoing work is assessing whether currently FDA-approved medications provide effective PEP or treatment for SEB. One of the most promising lines of research focuses on antiinflammatory and immunosuppressant agents as well as antioxidants. In particular, the immunosuppressant rapamycin (also known as sirolimus) has protected mice from intranasal and systemic exposure to SEB.[443] A recent study in a murine model of SEB-induced lethal shock found that 75% of

mice receiving a combination of the antiinflammatory drug dexamethasone (at 2 and 5 hours after SEB challenge) and the antioxidative drug N-acetyl cysteine (at 24, 30, 48, 54, 72, 78, and 96 hours after challenge) survived; by comparison, only 10% of untreated mice survived.[444]

Postevent Countermeasures: Current Options

Treatment is limited to supportive care, which should focus on oxygenation and hydration; severe cases with pulmonary edema may require ventilation, vasopressors, and diuretics. At this time, no PEP is available; individuals potentially exposed to SEB should be closely monitored for symptoms of intoxication and treated accordingly.[14,66]

Ricin

Ricin is a protein toxin derived from castor beans (the seeds of the castor oil plant, *Ricinus communis*). Ricin, a cytotoxic lectin, consists of an A-chain, the toxic portion of the protein, bound to a B-chain, which serves to bind the toxin to surface receptors found on mammalian cells, enabling the A-chain to enter the cell. Once inside the cell, the A-chain inhibits protein synthesis, which ultimately results in cell death.[445–449] Ricin can be delivered by aerosol, ingestion, or injection.[450] Inhalation of ricin as a small-particle aerosol may produce pathological changes beginning within 8 hours, manifested as severe respiratory symptoms associated with fever and followed by acute respiratory failure within 36 to 72 hours. Ingestion of ricin may result, beginning within 3 to 20 hours, in severe gastrointestinal symptoms (nausea, vomiting, cramps, and diarrhea) followed by vascular collapse and death. Injection can result, beginning within 6 hours, in general weakness and myalgias, followed by vomiting, fever, multiorgan failure, and death.[14,66,448,449]

Vaccination

No vaccine is available, but several vaccine candidates are being studied.[451] Because passive prophylaxis with monoclonal antibodies in animals is protective against ricin challenge, the vaccine candidates are based on induction of a humoral response.[452,453]

The most promising development for a vaccine has been to genetically engineer the ricin toxin A chain (RTA) subunit to eliminate both its enzymatic activity and its ability to induce vascular leaking. The nontoxic RTA subunit has been demonstrated to induce antibodies in animal models and to protect mice against intraperitoneal challenge with large doses of ricin.[451] A pilot clinical trial in humans demonstrated that a recombinant RTA vaccine (RiVax), given as three monthly intramuscular injections at doses of 10, 33, or 100 μg (five volunteers at each dose), was safe and elicited ricin-neutralizing antibodies in one of five individuals in the low-dose group, four of five in the intermediate-dose group, and five of five in the high-dose group.[454] However, the antibody response was of short duration. More recently, a phase 1B trial of Alhydrogel-absorbed RiVax found positive titers of anti-RiVax antibodies in four of five volunteers receiving three 10-μg doses and four of four individuals receiving three 100-μg doses. All of the eight individuals who seroconverted still had positive titers on day 252; five of these individuals continued to exhibit titers on day 364. The vaccine appeared to be safe and well tolerated.[455] A recently developed heat-stable version of RiVax could extend the vaccine's shelf life at high temperatures, potentially simplifying storage and distribution.[456]

A ricin vaccine candidate (RTA 1–33/44–198 or RVEc) developed at USAMRIID demonstrated high relative stability to thermal denaturation, no detectable cytotoxicity, and immunogenicity in animal studies. The vaccine demonstrated protective immunity against aerosol challenge with ricin in rodents, rabbits, and NHPs. Additionally, no toxicity was observed in two animal models.[457–460] In a phase 1 escalating, multiple-dose study, this vaccine was found to be safe, well tolerated, and immunogenic in healthy adults who received three doses of either 20 μg (10 volunteers) or 50 μg (10 volunteers) of RVEc. Among 10 volunteers who received a single 100-μg dose, 2 individuals developed elevated creatine phosphokinase levels, which resolved without sequelae; no further vaccinations were administered at this dosage. Four individuals in the 50-μg group received a single booster dose, which was safe and well tolerated; all booster recipients developed a robust anamnestic response.[461] Further studies are planned to optimize dose, scheduling, and route of administration.

A ricin toxoid vaccine encapsulated in polylactide microspheres or poly(lactide-co-glycolide) microspheres and given intranasally was demonstrated to be protective against aerosolized ricin intoxication in mice. Both systemic and mucosal immune responses were observed, with high titers of antiricin IgG2a at 2 weeks postvaccination and still present and protective in mice 1 year later.[462] Oral vaccination of mice with the ricin toxoid vaccine encapsulated in poly(lactide-co-glycolide) microspheres was also protective against lethal aerosol ricin challenge.[463]

Treatment and Postexposure Prophylaxis

No therapeutic or PEP agent for ricin intoxication has been developed. Although passive immunoprophylaxis of mice can reduce mortality against intravenous or intraperitoneal ricin challenge if given within a few hours of exposure, passive immunoprophylaxis is not effective against aerosol intoxication.[452,453] The development of prophylactic and therapeutic medical countermeasures for ricin intoxication is challenging in part because ricin is taken up into cells rapidly[464] and has high enzymatic efficiency,[465] leaving a narrow treatment window.

Postevent Countermeasures: Current Options

Individuals who may have been exposed to ricin should be monitored closely. Diagnostic testing could include nasal swabs, sputum, and induced respiratory secretions for assay via polymerase chain reaction or antigen enzyme-linked immunosorbent assay (Ag-ELISA) and serum for baseline toxin assays via Ag-ELISA or polymerase chain reaction.[66] Treatment consists primarily of supportive care, such as oxygenation, maintenance of electrolyte balance, and hydration for inhalational exposure and gastric lavage, administration of cathartics, and volume replacement of fluid loss for gastrointestinal intoxication.[14,66,466]

SUMMARY

Although medical countermeasures are effective in preventing disease, the greater challenge is to develop a balanced approach that may provide preexposure and postexposure medical countermeasures to protect both military and civilian populations. Generally, military personnel undergo prophylactic vaccination against a broad array of endemic diseases as deployments into areas not travelled by the masses could be required without significant advance notice. In addition, the military has recognized the benefit of vaccinating troops for protection against exposure to a biological weapons release in a battlefield setting. However, vaccination of civilians in advance may not be feasible because of the larger host of potential biological threat agents in a civilian population and the infrequent occurrence of bioterrorism events expected in a civilian population.

Vaccine recommendations for the civilian and military populations must weigh the risks and benefits as well as the logistics of maintaining immunity with vaccine booster doses. More studies to assess the long-term medical effects of repeated vaccination with multiple vaccines are needed to assure civilian and military populations about the safety of the long-term use of vaccines. Protection of the public from bioterrorism will require the development, production, stockpile maintenance, and distribution of effective medical countermeasures for both prevention and treatment of illness, with careful forethought about the balance of preexposure and postexposure countermeasures. It is likely that the military will be involved with both the distribution of medical supplies and the management of bioterrorism events within the continental United States; therefore, military physicians must be properly trained and prepared for managing bioterrorism events.

Acknowledgments

The following individuals were the authors of the previous edition of this chapter and contributed to this edition: Janice M Rusnak, Ellen F Boudreau, Matthew J Hepburn, James W Martin, and Sina Bavari.

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Chapter 28

FUTURE PROSPECTS OF VACCINES AND ANTIBODIES IN BIODEFENSE

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SUMMARY

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INTRODUCTION

Novel and next-generation vaccine approaches are being developed in parallel with new generations of antibody-based therapeutics. Each of these approaches has advantages specific to the disease, timeline, development status, and therapeutic or treatment window of its targeted pathogen. Usually, a specific or broadly neutralizing antibody response is required to provide either innate protection or vaccine-mediated protection, but this is not always the case. The increasing demand for antibody-drug conjugates, vaccine and small-molecule synergistic effects, and vaccine prophylactic use highlights the need to develop these two strategies individually and in combination to discover the optimal forms of protection.

Edward Jenner's 1794 discovery of the cross-protection afforded by a cowpox virus to variola virus led to the first vaccine, and ultimately to the successful eradication of variola on May 8, 1980, when the World Health Assembly certified the world free of naturally occurring smallpox. In the early 1880s, Louis Pasteur led the development of live attenuated vaccines, and in the late 1890s Emil von Behring and Kitasato Shibasaburo developed serum therapy against diphtheria and other microbes. Serum therapy was effective, but the administration of large amounts of animal proteins often led to undesirable side effects such as serum sickness. Within the same time period, vaccine developments provided efficient protective active immunity against rabies, tuberculosis, typhoid fever, and diphtheria and tetanus toxins.

The use of serum therapy declined after the discovery of antibiotics in the 1940s. It was later recognized that the critical protection afforded by crude serum was linked to polyclonal antibodies (pAbs), the "magic bullets" imagined by Paul Ehrlich. The development of biotechnology during the 20th century allowed Georges Köhler and César Milstein to isolate the first murine monoclonal antibody by immortalizing B cells in 1975. Hybridoma technology revolutionized antibody therapeutics and was later enriched by immortalization of human peripheral B cells, direct cloning of variable genes into phage expression libraries, and creation of transgenic mice that carry human antibody gene loci.[1]

The growing problem of increased bacterial resistance to antimicrobials, together with the need for alternative strategies to treat infectious diseases resistant to therapeutics, has stimulated a renewed interest in antibody therapy in the fields of infection and intoxication within biodefense. Although the risk of antimicrobial resistance is acute, an agent selected for deliberate dissemination may also be selected or engineered for antimicrobial resistance to existing treatments. Antibody therapy may be one way to mitigate that risk. Furthermore, utilization of antibodies in combination with existing therapeutics may provide synergistic benefits.

The pathway for monoclonal antibody (mAb) use in infectious disease follows proof-of-concept studies utilizing species matched fractionated serum, the use of human convalescent serum, and the early success of pAbs. Although initial results are promising, only a few mAbs are biodefense-related; namely raxibacumab, which obtained approval by the Food and Drug Administration (FDA) in 2012 for treatment of inhaled anthrax. However, several biodefense-specific mAb cocktails are showing success in early clinical phase trials.[2] The FDA has approved antibody therapy for emergency use when no other therapeutic options are available.[3] Throughout this chapter, monoclonal antibodies will be abbreviated as mAbs. Some of these therapeutics are also abbreviated as rAbs, or recombinant antibodies, in other texts, defined by their selection or engineering, and expression utilizing a range of DNA-based molecular biology techniques. Here, novel antibody designs (ie, fusion proteins) will be identified by their construct.

Vaccine development has also greatly benefited from advanced biotechnology. New vaccines may reveal previously unrecognized or underappreciated pathways to providing protection against biological weapons. This chapter will first examine the possible future of vaccines in a biodefense perspective, then consider how antibodies may provide novel and efficient ways to protect soldiers.

TRENDS IN BIODEFENSE VACCINE DEVELOPMENT

The FDA has fully licensed only two biodefense-related vaccines (Table 28-1). The development of vaccines is generally an expensive undertaking; a single vaccine usually takes 10 to 15 years to reach licensure, at a cost estimated in the hundreds of millions of dollars.[4] The pharmaceutical industry's evaluation of the cost is not simple, but a recent rotavirus vaccine is estimated to have cost between $205 million and $878 million.[5] For vaccine manufacturing companies, the biodefense market is limited, and as defense budgets globally tend to decrease, decisions to develop novel vaccines will be carefully examined. However, healthcare authorities are increasingly recognizing the benefit and cost-effectiveness of vaccines, so vaccine research in biodefense makes sense.

TABLE 28-1

MAIN VACCINE STRATEGIES APPLIED TO BIODEFENSE AGENTS UNDER INVESTIGATION OR AUTHORIZED BY THE US FOOD AND DRUG ADMINISTRATION

Strategy	Vaccine	Agent	Manufacturer	Status
Live attenuated vaccine	Vaccinia virus	Smallpox	Sanofi (Paris, France)	Authorized by FDA
	Francisella LVS strain	Tularemia	DynPort Vaccine Co LLC (Frederick, MD)	Under investigation
	Stern strain	Anthrax	Colorado Serum Co (Denver, CO)	Not authorized by FDA
	EV76	Plague	Not currently manufactured	Not authorized by FDA
Recombinant vaccine vectors	c-Ad3-EBO	Ebola virus	NIAID-GSK (Brentford, UK)	Under clinical investigation
	VSV-EBOV	Ebola virus	NewLink Genetics (Ames, IA); Merck (Summit, NJ)	Under clinical investigation
Subunit vaccine	AVA	Anthrax protective antigen	Emergent BioSolutions, (Rockville, MD)	Authorized by FDA
	LcrV-F1	Plague	Dynport Vaccine Co LLC	Under clinical investigation

AVA: anthrax vaccine adsorbed
FDA: Food and Drug Administration
LVS: live vaccine strain
NIAID-GSK: National Institute of Allergy and Infectious Diseases-GlaxoSmithKline
VSV-EBOV: vesicular stomatitis virus-Ebola virus vaccine

The future of vaccine development can be seen as responding to two different demands: On one hand, health authorities are pushing for safer, well-defined, purified vaccines, including vaccines with minimalist compositions that are able to protect against a single agent. Future biodefense vaccines developed in this context will need to follow these requirements. On the other hand, defense authorities want vaccines effective against dozens of different agents, protective against pulmonary contamination, triggering a rapid immune response, easy to administer, easy to produce on a large scale, and stable enough for long-term storage. In addition to these specifications, development and production costs should be kept as low as possible. These two global trends are clearly antagonistic, but emerging technologies may help in the development of safer vaccines with a larger range of efficacy. This section will examine how emerging technologies may improve old vaccines or give rise to new, multivalent vaccines.

Live Attenuated Vaccines

Jenner's serendipitous discovery of an attenuated vaccinia virus (VACV)[6] was later rationally extended and developed by Pasteur to produce vaccines against anthrax[7] and later rabies.[8] Live vaccines have since proven to be very efficient, as evidenced by the eradication of smallpox,[9] the prevention and control of poliomyelitis[10] and yellow fever,[11] and, more generally, the control of infectious diseases throughout the 20th century. The only attenuation technique available before the 20th century was the time-consuming and rudimentary passage of virus in transformed culture cell lines or in atypical hosts. Ideally, live attenuated vaccines are composed of live virus or bacterium that establishes a mild infection at the site of replication, which the immune system then controls by mounting an immune response. Live attenuated vaccines use a weakened agent, allowing for a controlled infection, which is why these vaccines optimally activate immune effectors and are more effective than inactivated vaccines.[12] Live attenuated vaccines must respect a delicate balance. They should be active enough to be immunogenic, but also sufficiently attenuated to be safe. The present regulatory environment imposes strong limitations on the development of new live attenuated vaccines to limit the risk of wild type virulence reversion, especially for virus-based vaccines. Discussed later are promising technologies that may aid in the development of live attenuated viruses as safer vaccine candidates.

Replication-defective viruses represent the first approach to improving safety and limiting the risk of uncontrolled infection in an immune-compromised host. As mentioned previously, this attenuation technique has historically occurred through serial passage; for example, a VACV was passed more than 570 times in chicken embryo fibroblasts, leading to the production of an attenuated vaccine called modified vaccinia virus Ankara (MVA) in the 1970s.[13] Through passage, MVA lost approximately 15% of its genome, was rendered replication-defective, and thus has proven to be safe in large clinical studies. Capitalizing on sequencing technology, researchers have synthetically developed an MVA homolog on a Lister strain vaccine virus background by deleting five regions similar to the regions lost by MVA.[14] This demonstrates one way that genetic-based technology can help in the development of rational vaccines. These viruses, however, are less immunogenic than their parental strains, illustrating the delicate balance needed between immunogenicity and safety. Because they are defective in replication, these vaccines are too efficiently eliminated, leading to a poor immune response.

Codon deoptimization is a novel strategy for developing live attenuated vaccines against RNA viruses, which represent a unique challenge due to their high genetic instability. Numerous studies have shown how the low-fidelity, RNA-dependent RNA polymerase can allow a virus population to rapidly drift or potentially revert any mutation.[12] Mammalians, as well as many other organisms, have codon bias, using synonymous codons more frequently than others. This bias is probably correlated to the efficiency of translation, and observed in virus structural genes. Codon deoptimization in the genome capsid of poliovirus induced a profoundly attenuated strain that triggered a strong immune response in animals.[15] As a general strategy for other vaccine development, codon deoptimization offers the major advantage that the genome is not mutated; therefore, it does not affect the antigenicity of the proteins. Codon deoptimization has been applied to different RNA viruses[16–18]; however, more studies are needed to evaluate whether this promising approach is safe and applicable for large-scale production.

Another strategy for the production of live attenuated vaccines is using microRNAs (miRNAs), which are genetically encoded short RNAs that have tissue-specific or developmental expressions and that play a large role in gene regulation.[19] Researchers have inserted miRNA sequences into the poliovirus genome to restrict its replication in the central nervous system (CNS).[20] However, it cannot be ruled out that the virus, by replicating outside of the CNS, could insert mutations in the miRNA sequence and revert its virulence.

Finally, zinc-finger (ZF) nuclease-controlled live attenuated virus is another experimental strategy that may be exploitable in the future as a vaccine.[12] This strategy has used ZF domains coupled to other functional domains to produce novel transcription factors that increased or decreased gene transcription with promoter specificity.[21–23] In other studies, ZF domains were fused to a restriction enzyme nuclease domain, resulting in the cleavage of specific sequences of double-stranded DNA.[24–26] This strategy could be useful in vaccination efforts to prevent or eliminate persistent viral infections.

The first live attenuated bacteria-based vaccine for human use was developed against tuberculosis through 230 serial passages of *Mycobacterium bovis* over a period of 14 years on artificial medium by Calmette and Guérin.[27] Live attenuated vaccines have historically been developed by the empirical technology of passage, and used in humans against anthrax (in the former USSR)[28] and plague (in the former USSR and in developing countries where plague is endemic),[29] but they are not authorized in Western countries. *Francisella* live attenuated vaccine live vaccine strain is licensed by the FDA, but could be improved. Advances in molecular biology and a better knowledge of host–pathogen interactions will help in the future development of rational strategies for novel live attenuated bacterial vaccines.

Recombinant Vaccine Vectors

Viruses can be engineered as carriers of heterologous antigens. This was elegantly demonstrated more than 30 years ago using a VACV expressing the hepatitis B surface antigen (HBsAg), which induced a heterologous protective immunity against hepatitis B in chimpanzees.[30,31] Although very promising, this strategy has yet to lead to a licensed vaccine for use in humans. Many viruses have been tested for their utility as a vaccine vector through their capacity to express heterologous proteins and induce an appropriate immune response. The myriad virus genera used to express recombinant protective antigen (PA) of anthrax can be seen as a paradigm of the extensive possibility of molecular biology in vaccinology. Anthrax PA has been successfully expressed by members from very diverse families of viruses, including poxvirus,[32] rabies virus,[33] hepatitis B virus,[34] adenovirus,[35] and influenza virus,[36] as well as in baculovirus and vaccina virus recombinants.[37]

Interest in the virus vector strategy was recently tested by the quick development of vector vaccines to help control the 2014 Ebola virus outbreak in West Africa. A replication-defective chimpanzee adenovirus

type 3 vector (cAd3-EBO) vaccine has shown interesting preliminary results in phase I trials,[38] while a vesicular stomatitis virus-based vector will soon enter the clinical phase.[39] Only a limited number of viruses have successfully undergone large-scale manufacturing practices and clinical trials and may one day reach human licensure. These include poxviruses, adenoviruses, Alphaviruses, and Flavivirus.[40–42]

Poxviruses represent good vector candidates because they grow to high titers, are very stable when lyophilized, and can accept very large transgenes due to their large genome sizes.[43] Moreover, the issue of preexisting immunity is limited because the adult population born after 1980 is naïve, and the protection afforded by the vector against variola provides dual-purpose use in a biodefense perspective. This last point has been exemplified by the development of a bivalent vaccine of a VACV vector expressing anthrax PA that provides immunity against anthrax and variola.[32] MVA has been used as vector for multiple viruses, such as human immunodeficiency virus (HIV), influenza, and dengue, but it has been largely supplanted by avipoxviruses, especially canarypox, which have a better safety profile because they do not replicate in mammalian cells.[44] Canarypoxvirus-vectored vaccines have been developed against rabies and measles and studied in HIV.[44] Although they proved to induce significant immune responses, the immune response is reduced in hosts with preexisting cross-protective immunity against the vector. This relatively new field would benefit immensely from a greater understanding of precisely which viral genes are crucial to triggering an efficient immune response.

Human adenoviruses (Ads) are also live vectors well suited for vaccine development, with a large genome of 38 kb of double-stranded DNA.[41] Recombinant Ads vectors have the E1 segment deleted, allowing the insertion of 5 kb of heterologous antigens, and are replication-defective in human cells. Most Ads vectors have been developed using Ad5, Ad26, and Ad35.[41,45,46] Ad5 vectors can be administered by nasal spray, allowing targeted vaccination of the mucosal surface. The main drawback of Ads vectors is the presence of preexisting immunity for Ad5 and the recombination hazard during the production process.

Another viable vector platform is virus-like replicon particles (VRPs). VRPs have been generated using the members of the Alphavirus or Vesculovirus genera. Alphaviruses contain a single-stranded, positive RNA of 12 kb and are members of the family *Togaviridae*. Replicons derived from Sindbis virus, Semliki Forest virus, and Venezuelan equine encephalitis virus (VEEV) were engineered by replacing structural genes with heterologous genes.[47] Vesicular stomatitis virus-based VRPs expressing Marburg virus glycoprotein-induced immune cross-protection against various strains of Marburg virus.[48]

Yellow fever virus (YFV) was developed under the ChimeriVax technology by Sanofi (Paris, France) as a chimeric virus for other Flaviviruses, such as dengue, West Nile virus, and Japanese encephalitis virus.[42] The platform consists of the live YFV 17D vaccine backbone virus deleted of its premembrane and envelope genes, which were replaced by heterologous genes from other Flaviviruses. This vector has been closely examined regarding its safety and the risk of recombination in the environment. Although safe and tested in large phase III clinical trials for dengue, this technology is limited to the *Flaviviridae* family.[49]

Bacteria can also be used as a vector for carrying vaccine antigens. *Listeria monocytogenes* and *Salmonella* species have been used as vaccine vectors in numerous clinical trials, mainly as cancer vaccine candidates.[50] *Salmonella* has shown some promise from a biodefense perspective as a vector for vaccines against pathogens such as anthrax[51] and plague[52]; however, for a vaccine strategy in a general population, less pathogenic strains are desired.

Among many models, lactobacilli are regarded as interesting bacteria because they can induce a mucosal immune response by expressing an antigen locally. It has been shown that PA from *Bacillus anthracis* expressed by a *Lactobacillus gasseri* induces significant protection against the infection in mice.[53] Because lactobacilli are probiotic, they can be administered easily and carry multiple genes, providing potential immunity against diverse agents.

Additionally, *Bacillus* spores can be used as an antigen carrier and vaccine platform.[54] This platform is promising because the spores are very stable and can be used as a mucosal vaccine. As an example, *Bacillus subtilis*-expressing PA has been shown to induce protective immunity against anthrax in a murine model.[55]

One of the main hurdles for vaccine vectors is the evaluation of efficacy in a human population that may have acquired various levels of preexisting immunity. Limited data has been published so far on this topic, but a recent review of the literature suggests that for *Salmonella* vectors preexisting immunity can enhance subsequent induction of immunity, while for adenovirus preexisting immunity is a hindrance.[56]

Subunit Vaccines

Since the inception of vaccinology, the hunt for ways to address attenuated vaccine safety concerns has resulted in the search for immunogenic subunits. This was first exemplified in the 1920s by the

discovery that formaldehyde-inactivated exotoxins are immunogenic.[57] However, beyond exotoxins, large-scale production of subunit antigens was not feasible at the time. During the "golden age" of vaccinology, in the 1960s and 1970s, most vaccines were inactivated viruses or bacteria. Renewed interest in the development of subunit vaccines occurred in the 1980s with the emergence of a hepatitis B virus subunit vaccine composed of the HBsAg protein, initially purified from patient sera and later produced by genetic engineering.[57] Subunit vaccines are attractive for use in biodefense because they offer stimulation of targeted immune responses with minimal side effects. Indeed, the anthrax PA-based vaccine BioThrax (Emergent BioSolutions, Rockville, MD) and next-generation plague fusion protein LcrV-F1 vaccine currently under clinical investigation are both antigen-targeted subunit vaccines produced through genetic engineering.

First demonstrated 35 years ago, synthetic virus-like particles (VLPs) are molecular structures based on viral protein subunits that form artificial capsid units.[58] VLPs mimic native virions by displaying repetitive subunits of the viral surface proteins that present the immune system with conformational viral epitopes that can elicit strong T cell and B cell immune responses; however, they can be distinguished from virus by the absence of nucleic material. The absence of replication renders them safe antigens for use as a vaccine. In 1986, at the dawn of recombinant technologies, HBsAg was produced with yeast to form the first synthetic type of VLP to come on the market (Recombivax B [Merck, Summit, NJ] and Engerix B [GlaxoSmithKline, Brentford, United Kingdom]). Some 20 years later, two human papilloma virus (HPV) vaccines (HPV 16 VLP vaccine and HPV 18 VLP vaccine)—based on the L1 protein produced in yeast or insect cells—were licensed (Cervarix [GlaxoSmithKline] and Gardasil [Merck]).[59] More recently, a VLP-based hepatitis E virus vaccine was licensed for use in the Chinese market (Hecolin [Xiamen Innovax Biotech, Xiamen, China]).[60]

Numerous VLP-based vaccines are currently in preclinical development and clinical trials, which shows the potential of this strategy.[61] Of particular interest for biodefense, VLPs have shown promising initial results in protection against the Ebola and Marburg viruses.[62] VLPs consisting of up to three antigens—(1) the glycoprotein, (2) the matrix protein (VP40), and (3) the nucleoprotein—as well as "hybrid VLPs"—containing both Ebola and Marburg glycoprotein and nucleoprotein—can confer protection against homologous challenge with either virus in rodents and nonhuman primates.[62] VLPs can be genetically engineered, incorporating peptides, proteins, or pathogen-associated molecular pattern ligands to create elaborate scaffolds.[63]

The plasticity and potential of VLPs are quite remarkable and limited only by the human imagination. Their plasticity is exemplified by insect Flock House virus VLP nanoparticles that display anthrax antigen. When the VLPs are coated with the PA-binding domain of anthrax toxin receptor 2, they serve as a decoy receptor for anthrax toxins in vitro and in vivo. Alternatively, when coated with PA protein, the VLPs can be used as an efficient anthrax vaccine.[64] VLPs can be used as a molecular platform to help the immune system recognize heterologous antigen. These characteristics place synthetic VLPs at the boundary between subunit antigen and adjuvant (see the discussion under Adjuvants).

Initially launched in the 1980s with the first pneumococcal vaccine, recent technological advances in glycan synthesis, glycan structure analysis, and glycochemistry are paving the way for a new era in carbohydrate vaccine design.[65] In this approach, the surface of bacteria is covered by a dense array of polysaccharide on the lipopolysaccharide and the capsule, forming a unique feature with strong antigenic properties. It is important to note that glycan synthesis is complex and its support of antigenic specificity is not genetically encoded. The conjugation of glycans to a carrier protein or to an adjuvant induces long-lasting protection against encapsulated bacteria, which could potentially be extended to viruses. This novel and booming field may help develop new vaccines against gram-negative or capsulated bacteria such as *Yersinia pestis*, *Francisella tularensis,* and *Burkholderia pseudomallei*.

Advances in genomics, including high-throughput DNA sequencing, have provided access to complete antigenic repertoires of pathogens. Capitalizing on this information, in a process coined "reverse vaccinology," previously unknown vaccine antigens have been identified.[66,67] The first success story of this strategy was the long-sought vaccine against meningococcus B.[68] In contrast to *Neisseria meningitidis* serogroups A, C, Y and W135, carbohydrate vaccines could not be used for serogroup B due to the similarities between the capsular polysaccharide and a human neural antigen. The first step of the lengthy process of developing this vaccine was a computer analysis of the targeted genome sequence to identify all genes coding for predicted antigens. The 600 predicted antigens were screened for their expression by the pathogen and their immunogenicity (referred as the antigenome).[69] Twenty-nine selected antigens were further tested for their immunogenicity in animal

models. Lastly, the selected PAs were evaluated for their presence in worldwide representative pathogen collections. Five stable PAs were industrially produced for clinical trials. At each step of the process, the number of antigens dramatically decreased, but the power of the strategy comes from the complete genome analysis.[68] Although this strategy is costly, it may be applied to diverse biothreat agents for which other methods of vaccine development have so far been ineffective.

"Systems vaccinology" is a generic term recently coined to describe the use of systems approaches to identify signatures that can be used to predict vaccine immunity in humans.[70] The two major goals of systems vaccinology are to characterize the host response by identifying genes and pathways whose expressions are altered in those receiving vaccines, and then to identify predictors of vaccine efficacy. Systems vaccinology is still in its infancy, but it may help in the future design of efficient vaccines, especially for pathogens that constantly change their surface-expressed molecules, such as plasmodia and other eukaryotic parasites.[71]

Structural vaccinology, the use of three-dimensional structural information to design novel and improved vaccine antigens, is another trend in rational vaccine design. Advances in X-ray crystallography and nuclear magnetic resonance spectroscopy have enabled researchers to pinpoint new structures and antigenic epitopes at atomic resolution.[72] Recent advances may have solved one of the hottest issues in vaccinology: respiratory syncytial virus (RSV). RSV has been a challenge for vaccine development after a disastrous clinical trial in the 1960s showed that a killed vaccine enhanced the disease, increasing hospitalization rates of children and causing two deaths.[73] RSV fusion (F) glycoprotein, which helps the virus merge its membranes with the host cell, exists in two conformational states, the prefusion metastable state and the postfusion stable state.[74] By engineering soluble variants of RSV F with a stably exposed antigenic site, researchers have identified one easy-to-produce variant that induces a strong neutralizing immune response in murine and nonhuman primate models.[75] Complete understanding of antigenic structure may aid the identification of key sites to target in order to disable a protein's function, stabilize select conformations of a protein, or determine antibody–antigen complexes, all of which would allow the development of more stable, homogeneous, efficiently produced vaccine antigens. This strategy might be applied with great success to viruses for which neutralization could be obtained through surface glycoproteins, such as hemorrhagic fever viruses.

Nucleic Acid Vaccines

Proof-of-concept studies for nucleic acid vaccines occurred more than 2 decades ago, when it was shown that intramuscular injection of mRNA or DNA resulted in local production of a reporter gene[76] and the induction of an immune response.[77,78] For DNA vaccination with this approach, the gene of an antigen is encoded and expressed from a plasmid-based system. In theory this method provides a subunit vaccination; however, by expression of the antigen within the host, rather than exogenous supplementation of the protein, the antigen is expressed in its natural form. As a result, the antigen can be processed by the immune system to activate both humoral and cellular immune responses.[79] DNA vaccines are particularly attractive in the biodefense field because they offer many advantages: (*a*) they can be engineered without the need to culture a pathogen; (*b*) manufacturing processes to produce plasmid DNA are well-established; (*c*) plasmid DNA manufacture is extremely rapid and can be designed for any engineered or emerging pathogen; and (*d*) the vaccines are safe and pose no risk of integration into the genome.

Despite these obvious advantages and many clinical trials, no DNA vaccines have been authorized for human use by the FDA.[80] Many hurdles need to be overcome if there is to be a future for this vaccine class. One such challenge is the manufacturing of plasmid DNA on a large scale with good manufacturing processes.[81] In the biodefense field, DNA-based vaccines have been tested against anthrax with somewhat disappointing results, but they have shown efficiency against Filoviruses, poxviruses, and encephalitic Alphaviruses.[82] The greatest challenge, though, is the suboptimal immunogenicity elicited by the vaccines in humans, which has been found to be significantly lower than that observed in rodents. Several strategies have been developed to improve the vaccines' immunogenicity.[79] As with live vaccines, codon optimization has been tested and helps with transgene expression in human cells. Another strategy has been to optimize the design of the transgene, including adding untranslated regions which may be critical regulators of vaccine gene expression located 5′ and 3′ to the encoded gene. One additional strategy has been to include unmethylated cytosine-phosphate-guanine (CpG) motifs in the sequence. CpG motifs are absent from eukaryotic DNA and are recognized as pathogen-associated molecular patterns by toll-like receptor (TLR) 9. The presence of CpG motifs may increase immunogenicity by stimulating a robust innate immune response.

In addition to making alterations in the plasmid DNA itself, changes in the delivery of DNA vaccines may enhance the quality and magnitude of the immune

response elicited. Many technologies have been tested, and the debate over their efficiencies is as long-standing as DNA vaccine technology itself.[83] So far, needle injection (subcutaneous or intramuscular), particle bombardment ("gene gun"), high-pressure liquid delivery, and electroporation have been evaluated, and each has specific advantages and disadvantages.[83,84]

RNA vaccines have been developed more recently, mainly because RNA molecules were known to be less stable. Despite this instability, RNA vaccines retain three major advantages over DNA vaccines.[85,86] First, RNA must be delivered only into the cytoplasm of target cells (in contrast to DNA, which must be transported to the nucleus). Second, genomic integration of the RNA is not possible, circumventing this safety concern (as well as the risk of inducing anti-DNA antibodies). Thus, RNA vaccines are not classified by the FDA as "gene therapy." Third, RNA interactions with the host are very versatile because the intracellular host innate immune response is focused on the detection and elimination of exogenous (mainly viral) RNAs. Due to the transient nature of RNA and the requirement of translation by host machinery, the load of antigen produced is more controlled, thereby minimizing the risk of tolerance induction by long-term antigen exposure.

Proof-of-concept studies demonstrating the efficiency of RNA vaccines has been established for numerous antigens.[85–87] However, RNA vaccines are still very experimental and require improvements to overcome the hurdles of clinical vaccine development. RNA stability and delivery methods need to be improved. Ribonucleases are present on the skin and in tissues, and mRNAs are negatively charged, which impairs their entry in the cytosol through the cell membrane. New methods for improving the RNA transfection efficiency may help. A recent study has shown that encapsulating a self-amplifying RNA into a lipid nanoparticle protected it from ribonuclease digestion and elicited a broad, potent, and protective immune response in rodents.[88]

High production costs and low RNA yield from in vitro production of mRNAs presents the second major challenge to the development of RNA vaccines.[89,90] So far, the longest chemically synthetized RNA with biological activity is 117 nucleotides.[90] Next-generation nucleic acid vaccines will also require an improved delivery technology, assessed in conjunction with the development of the vaccine.

Mucosal Vaccines

During an attack, biothreat agents would likely be presented in one of two primary routes of administration: aerosols or introduction into food or water supplies. Thus, an infectious agent would enter the host through the respiratory or intestinal mucosae. For this reason, vaccines targeting the mucosal surfaces seem logical for biodefense. However, a distinction between agents that elicits mucosal infections and those that simply exploit mucosal tissues as a mean to gain access to the body should be made.[91] When developing a vaccine to protect the mucosal surfaces, two strategies exist: (1) to increase the mucosal response of existing systemic vaccines and (2) to design a vaccine targeted for mucosal delivery and immune response,: the latter will be examined in this section. Mucosal vaccine development should be focused on agents that provoke a mucosal-associated infection, but not on agents that are controlled by systemic immunity. Mucosal vaccines offer many advantages from a production and regulatory point of view[92]: (*a*) oral vaccines do not need purification steps because the gut microbiota is already complex; (*b*) mucosal vaccines do not require injection, so they are subject to a better compliance and are suited for mass vaccination as they do not require medical personnel for administration; and (*c*) mucosal vaccines do not carry risk of spreading blood-borne infection because no needles are involved.

Mucosal immunology is an expanding field that has led to a better understanding of the mucosal immune system and response. Nevertheless, few mucosal vaccines have made it to licensure. Thus far, the only live attenuated virus vaccine administered intranasally are FluMist (MedImmune, Cambridge, United Kingdom), against influenza virus, and Vaxchora (PaxVax, Redwood City, CA), a suspension of *Vibrio cholerae* strain CVD 103-HgR) against cholera.[93] Two other live attenuated vaccines administered orally against rotavirus[94] and *S typhi* have also been efficiently launched.[95] Notably, two inactivated oral vaccines against cholera have been marketed, providing an interesting proof-of-principle for future inactivated vaccines.[96] In the biodefense field, *Y pestis* has been a model for the development of live mucosal vaccines providing protection against bubonic and pulmonary plague.[91]

A major challenge for mucosal vaccine development is the production of candidates that strike a balance between immunogenicity and attenuation. Historically, most mucosal vaccines have been produced through passage of virus in host cell culture, as was done for the oral polio vaccine. It is clear that mucosal vaccines will benefit immensely from a more rational approach using genetic manipulation to increase their safety and stability, as exemplified by the typhoid Ty21A vaccine.[97]

Another area of mucosal vaccine development that needs attention is characterizing the optimal route of immunization. The biodefense community frequently

focuses on intranasal, oral, and sublingual routes of immunization. The mucosal immune system is compartmentalized into nasopharynx-associated lymphoid tissues, bronchus-associated lymphoid tissues, and gut-associated lymphoid tissues.[98,99] Although some functional connections between the respiratory and gut immune systems have recently been described,[100] the presumably limited connectivity between the respiratory tract and the gut places a constraint on the definition of the optimal route of immunization. As a result, different routes of immunization tend to result in compartmentalized responses, with (*a*) intranasal immunization inducing a strong immune response (measured by the secretory immunoglobulin A [IgA] production) in the upper and lower respiratory tract and the gastric and genital tracts; (*b*) sublingual immunization inducing a response in the upper and lower respiratory tract and the gastrointestinal tract; and (*c*) oral immunization triggering an immune response in the salivary glands, gastrointestinal tract, and mammary glands.[98,99]

Development of a strong immune response to oral vaccination is especially challenging because of oral tolerance and the host's need to maintain homeostasis to protect against immune responses to digested antigens. Even after preclinical success with oral vaccination, many clinical studies have failed to induce an effective immune response.[97,99] Therefore, oral vaccine formulation studies are needed to gain a better understanding of the effects of stabilizing the antigens in a harsh environment and targeting them to the gut-associated lymphoid tissues. Intranasal administration remains attractive because of the large mucosal surface area the nose provides and the access to the nasopharynx-associated lymphoid tissues, which can activate immune responses in the respiratory and gastrointestinal tracts.[99] The seemingly global mucosal stimulatory potential after intranasal immunization may represent an important path for novel vaccines. Moreover, intranasal administration lowers antigen and adjuvant doses compared with oral vaccinations, making them more cost effective. Aerosol spray, droplets, and powders have been optimized and represent an attractive field for the development of new devices.[101,102]

However, the primary role of the nasal mucosa is to protect the respiratory airways, not to convey antigens to the immune system.[101] A challenge for intranasal immunization is that the nasal mucosa is intricately connected to the olfactory nerve and the CNS. As a result, intranasal immunizations using *Escherichia coli* heat labile toxin as an adjuvant in humans has been correlated with Bell's palsy development.[103] In contrast, sublingual immunization has gained interest because it stimulates a broad activation of the immune system while avoiding perturbation of the CNS.[104]

Adjusting mucosal vaccine formulations may be another way to improve their efficacy. Little is known about how to formulate a better mucosal vaccine, although there are currently two main approaches: using either (1) soluble or (2) particulate vaccines.[92] Defining the most effective vaccine formulation is a universal challenge in vaccinology (see the discussion in Adjuvants).

Finally, the development of new mucosal adjuvants could significantly improve the effectiveness of mucosal vaccines. Stimulating the appropriate type of immune response can dramatically affect the immediate and long-term immune response to a vaccine, ultimately determining protection from disease. The use of heat labile enterotoxin and cholera toxin has been abandoned due to neurologic effects and overt diarrhea, respectively.[105] In the future, mucosal adjuvants may be improved by combining particles with TLR ligands.[106] Alternative adjuvants are discussed in the following section.

Adjuvants

The word "adjuvant" is derived from the Latin verb "adjuvare" meaning "to help." Adjuvant properties were discovered by Gaston Ramon in 1920.[107] Although adjuvants have proven to be crucial for most vaccines, the field has been relatively neglected until recently. The most widely used adjuvant to date, alum, has been empirically instilled in billions of vaccine doses since the 1920s, even though its mode of action was not discovered until 2008.[108] The only other category of adjuvant authorized for human use is the squalene derivatives: MF59 by Novartis (Basel, Switzerland),[109] AS03 by GlaxoSmithKline,[110] and AF03 by Sanofi-Pasteur (Lyon, France).[111] These adjuvants have been used in the pandemic influenza vaccine since 2009.[112] Over the past decade, many preclinical studies have expanded the list of potential adjuvants.[113,114] Historically, adjuvants have been recognized as a "perfect mix" of old ingredients, including water in oil (w/o) and oil in water (o/w).[115] Their adjuvant properties are thought to be based on nonspecific inflammatory stimulation that brings all the cellular players to the site of inoculation. Instead of the typical adjuvant formulations of the past, some large pharmaceutical companies such as GlaxoSmithKline have developed new mixes adapted for specific vaccines that require strong and long-lasting immune responses.[116] The rediscovery of the central role the innate immune response plays in the

development of overall immunity has encouraged the design of TLR and nucleotide-binding oligomerization domain receptor (or NOD-like receptor [NLR]) ligands as adjuvant components. For example, AS04, developed by GlaxoSmithKline, is composed of the TLR4 agonist 3-*O*-desacyl-4′-monophosphoryl lipid A (MPL), a component of lipopolysaccharide.[116] Interestingly, this bacterial component has proven to be a good adjuvant for virus vaccines such as the human papilloma virus vaccine Cervarix, demonstrating that viral and bacterial motifs can cooperate to activate the immune system. More intricate mixes have also been tested by GlaxoSmithKline, including AS02, which contains MPL and QS-21, a saponin-based adjuvant derived from the bark of *Quillaja saponaria*.[116]

In the same context, there is potential that the emerging nanotechnology field may help improve adjuvants in the future.[63] Nanoparticles (1–1,000 nm size particles) retain adjuvant activity by improving antigen delivery and triggering innate immune responses. Numerous polymer motifs, such as poly(lactide-co-glycolide) nanoparticles, co-polymers hydrogels, and cationic liposomes, have been tested as carriers of antigens. Some biodegradable nanoparticles are safe and already available for use in vaccine formulation.[117,118] These particles can be decorated by molecular motifs either to target specific cells, such as dendritic cells or macrophages, expressing specific pattern-recognition receptors. Nanotechnology may supply an opportunity to improve vaccine adjuvants used in biodefense.

THE FUTURE OF ANTIBODIES IN BIODEFENSE

Just as with vaccine discovery, the development of antibody-based therapeutics can be an expensive and time-consuming effort, with new products taking years to reach licensure; however, most of the more recent products are of human or human-like origin, often requiring less time than older products to reach this milestone. Antibody-based therapeutics represent the fastest growing class of biological therapeutics: 43 therapeutic mAbs had been approved as of December 2013, and 36 of these are still active in the European Union or US market.[119] However, few of these are specific to biodefense agents. Biodefense-specific infectious agents and toxins are generally not as well studied, limiting the targets and antigenic material required for antibody generation. The production of these therapeutics has predominantly been hindered by the historically high cost of antibody therapeutic development, primarily in the production of a final protein in the concentration and format necessary to elicit protection. Also, in contrast to the "one-bug, one-drug" approach often used for vaccines designed to combat multiple agents (utilization of a single drug for each agent), antibody development costs may be increased by the need for oligoclonals, or cocktails, of multiple antibodies that focus on a single agent, often acting via different mechanisms or protecting against escape mutants. Despite these constraints, antibody therapeutics are increasingly necessary to fill the gap when vaccine development has yet to produce an efficacious product, as in the 2014 West Africa Ebola virus outbreak.[120]

Antigen-specific protection afforded by antibodies can have advantages compared to the protection elicited by vaccines, especially for biodefense. Vaccines must elicit an effective, long-lasting immune response whose maintenance may require multiple booster injections. Antibodies, however, may be administered in quantities that achieve a titer of protection that exceeds that elicited by vaccines due to the decreased immunogenicity of antibodies when used in a human or humanized format. These mAbs may then provide a higher level of protection, a level necessary in biodefense, because bioweapon exposure is often intended to use elevated levels of the agent or toxin compared with natural exposures. Emerging diseases and toxins do not always represent a threat exclusively, and several agents have been evaluated for their therapeutic potential. For example, botulinum neurotoxins (BoNTs) have a therapeutic application, making vaccination clinically disputable. Antibodies can serve multiple roles: as a therapeutic alternative when no vaccine is available or as the sole treatment when a vaccine would not be efficacious. This section will discuss new and emerging technologies that have improved antibody discovery; events specific to antitoxin, antibacterial, and antiviral antibody development; and the future formats and production challenges of these molecules.

Antibody Generations and the Development of New Therapeutic Formats

The most representative and recognizable antibody format, immunoglobulin G (IgG), is often the simple fractionation or isolation of antibodies from human or animal sera to produce protective antibodies (Figure 28-1). Additional antibody formats will be discussed later in this chapter.

Sera, the first antibody product used, has recently seen a resurgence of use. Generally, sera is fractionated to produce pAbs that are used as a first line of defense

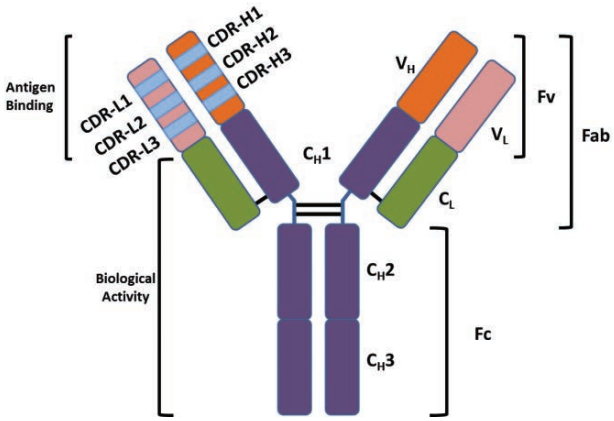


Figure 28-1. Representation of the immunoglobulin G antibody isotype. Specific regions are identified by color. The variable light (V_L) and variable heavy (V_H) regions, in *pink* and *orange*, respectively, together make up the Fv region. Each Fv and C_H1 domain (upper *green* and *purple* for each V domain) makes up a Fab fragment. The combination of the C_H2 and C_H3 (*purple*) domains makes up the Fc fragment. Fab: fragment antigen-binding; Fc: fragment crystallizable; Fv: variable fragment

against emerging pathogens; pAbs have been essential to providing critical treatments for infectious diseases. The use of these pAbs, as well as their role as future therapeutic countermeasures, will be discussed later within each subsequent section.

Historically, mAbs have been associated with several advancing generations of molecules (Figure 28-2). The first generations of mAbs were strictly murine in origin, as described in the early 1980s by Köhler and Milstein, who used hybridomas from stable fusions of immortalized myeloma cells with B cells from immunized mice.[1] Several of these mAbs made it to clinical trials for the treatment of cancers and transplant rejection, but were ultimately withdrawn due to a variety of concerns, including the high immunogenicity of these foreign proteins in humans, first identified by responses from human anti-mouse antibodies.[121]

Due to the immunogenicity of these antibodies, murine mAbs had little utility and often caused adverse effects. Following the publication of Köhler and Milstein's hybridoma article, human/mouse chimeric antibodies were developed as second generation therapeutic molecules with a human Fc region to decrease immunogenicity.[1] Several biodefense mAbs have been produced in this format, primarily due to the ability to develop antibodies using mouse models for exotic infectious diseases that could be rapidly chimerized to a human Fc region for a single clinical application. Even with the addition of a human portion to the antibody, the administration of these chimeric antibodies continued to produce an immune response to the remaining murine domains.

Third- and fourth-generation mAbs were developed by using selection and engineering advances in recombinant DNA-based molecular biology techniques to reduce immunogenicity. This was accomplished through humanizing the framework regions and transferring only the antigen-binding loops made up by the complementary-dependent regions. Further humanization can be achieved by making point mutations within the complementary dependent regions themselves. Humanization of each mAb generation requires special care because multiple residues of the framework regions are often required for stability of the antibody; therefore, it is essential to ensure that these modifications do not negatively impact the binding or activity of the mAbs when changing residues within the complementary dependent regions. Although the overall immune systems of mice and

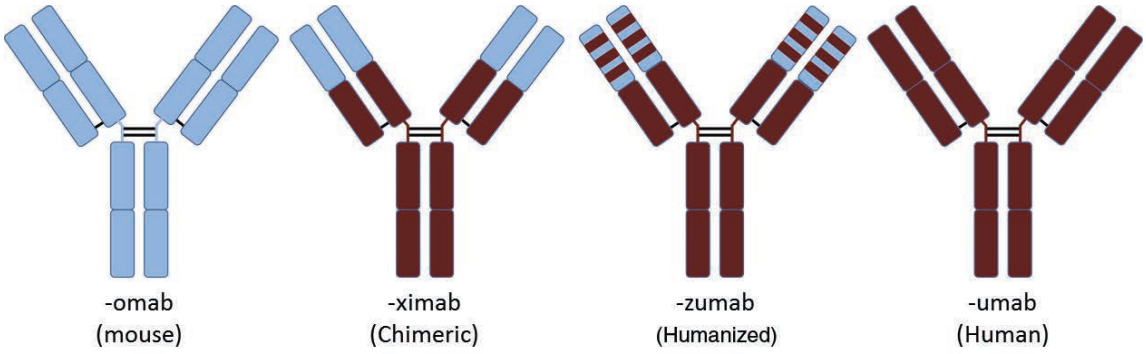


Figure 28-2. Representations of the progression of antibodies through the progressive generations of humanization from mouse (-omab), chimeric (-ximab), humanized (-zumab), and human (-umab) antibodies.

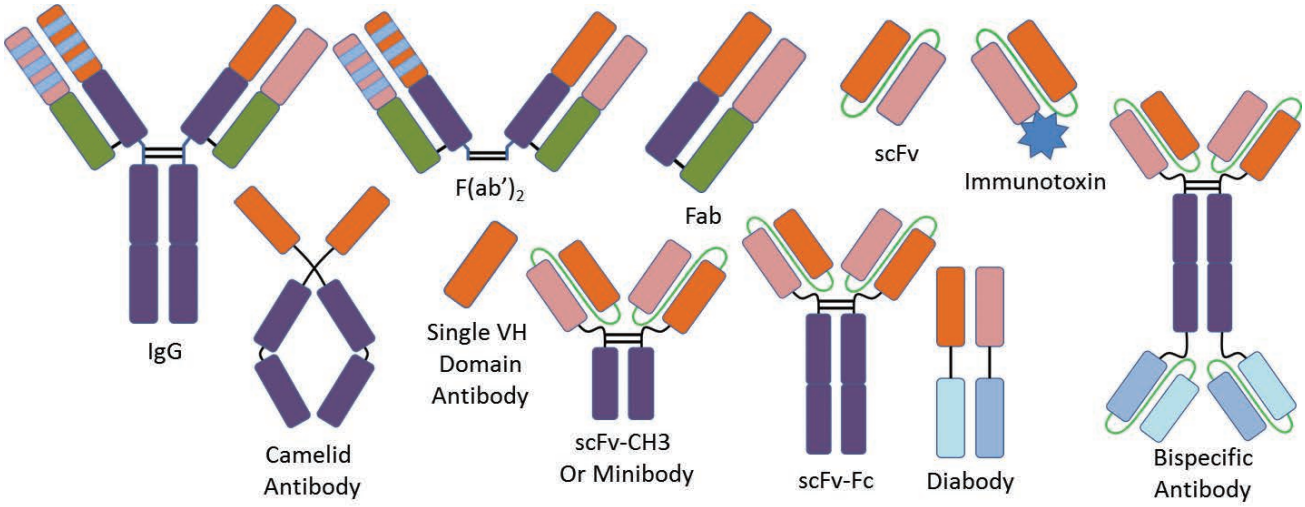


Figure 28-3. Representative antibody fragments demonstrating therapeutic potential through particular function, access, or host interaction. Immunoglobulin G (IgG) shown on *left* for reference.
Fab: fragment antigen-binding; Fc: fragment crystallizable; scFv: single-chain variable fragment; VH: variable heavy
Illustration: Adapted with permission from Frenzel A, Hust M, Schirrmann T. Expression of recombinant antibodies. *Front Immunol*. 2013;4:126. This work is licensed under a Creative Commons Attribution 3.0 Generic License (https://creativecommons.org/licenses/by/3.0/legalcode).

humans are similar, researchers should understand that the response in mice might not always correlate to the human response.[122] The most recent techniques used to produce human or humanized mAbs include DNA-based selection libraries (eg, phage display[123]), isolation from peripheral blood B cells,[124] and use of transgenic humanized animals.

The future of antibody development may not be in the isolation of the antibody to obtain a fully human product, but rather in the production of new formats that are able to enhance a particular antibody function, or in the administration of the antibody to the target or host. New mAb therapies being explored use not only antibody–vaccine combinations, but also antibody–drug conjugates[125] and many new antibody-based formats, such as bispecific and multispecific antibodies,[126] intrabodies,[127] and transbodies[128] (Figure 28-3).

The IgG1 isotype, depicted in Figure 28-1, is the most abundant isotype of immunoglobulin produced against protein antigens. Other isotypes include IgA, IgD, IgE, IgM, and multiple subclasses of IgG. As therapeutics, IgGs have seen the greatest use because they are the most predominant and abundant antibody in the serum and have historically been easier to isolate and purify. IgMs are large-molecular-weight pentamers and are the first low-affinity antibodies expressed in response to an infection. These antibodies are secreted from the surface of B cells before their maturation into a plasmocyte prior to the IgG isotype switch. The lack of recombinant and isolated IgMs available as therapeutics may predominantly be due to their overall molecular weight and the subsequent difficulty of purifying them.

IgAs consist of monomers in human serum and dimers after secretion at the mucosal surface through polymeric Ig receptors, are most prominent within the mucosal and gastrointestinal tract and serve as the first line of defense against natural infections.[129] The lack of alternative antibody isotypes beyond IgGs available for use against therapeutic targets can partly be attributed to the lack of appropriate in vivo models and partly to the difficulty in purifying these products in their dimer form. New transgenic species and advanced antibody production methodologies will most certainly provide the necessary tools to test alternative classes of antibody therapeutics.

IgG antibody isotypes, and to a lesser extent, fragment antigen-binding (Fab) fragments, have been the primary therapeutic format of antibodies that have been FDA approved for use. These antibodies have an extremely large size (around 150 and 55 kDa, respectively) and a half-life averaging 21 days for a human antibody, but they may not have optimal presentation to the epitope or tissue. Functional CH2 domains (called nanoantibodies),[130] single-domain heavy chain (abbreviated VHH) camelid-derived nanobodies,[131] and fusion antibody fragments are progressing through late stage development and into clinical trials. As in the case of single domain nanoantibodies and nanobodies, these smaller, independently folded domains with antigen

binding capabilities may serve as highly specific and extremely stable therapeutics. Although they possess remarkably stable physiochemical properties, these antibodies are rapidly cleared from the bloodstream in a matter of hours, hindering their development as a potential therapeutic in their base format. Methods to increase the half-life of these antibodies include binding and linkage to Fc domains, albumin, and polyethylene glycol. For both full-length antibodies and new single-domain formats, a range of methods is being developed to increase the antibodies' serum half-lives.

Antitoxin Monoclonal Antibodies

Treatment for intoxication using mAbs has seen a resurgence of utility in the past several years. Early work with serum therapy and passive transfer of antibodies provided successful proof-of-concept studies against diphtheria and tetanus via toxin neutralization.[132] Subsequent development of antitoxin antibodies, using high antibody affinity as a primary discriminator for selection, has shown advances against bacterial and viral targets.[133–135] Selection for these affinities has spurred vast improvements in anti-ricin and anti-BoNT, often into the sub-nM and pM levels. However, the narrow specificity of the antibodies to the toxin targets often limits their efficacy when used against toxins with multiple serotypes, as with BoNT, which has seven known serotypes (A through G).[136] Selection of these antibodies has been further driven by improvements in humanization. This section will primarily focus on toxins that have the capability for weaponization as biowarfare toxins in the absence of the producing pathogen.

Ricinus communis, a plant extensively cultivated around the world produces the Ricin toxin. Ricin is a type 2 ribosome-inactivating protein consisting of two parts. The B-subunit (RTB) binds to sugars on the cell surface for cytosolic entry of an A-subunit (RTA), which inhibits protein synthesis. Both of these subunits of the toxin have epitopes that have been targeted for therapeutic and vaccine development. Although two vaccines are undergoing early phase clinical trials (NCT01317667 and NCT00812071), there are currently no licensed measures for treatment, leaving options for supportive care only. (See Table 28-2[137–145] for more details.) A proof-of-concept study using direct inhalation of ricin toxin followed by treatment with pAbs 20 minutes postchallenge demonstrated protection.[146] Monoclonal antibody development has progressed through multiple antibody formats against both RTA and RTB.[147,148] Chimeric mouse/human antibodies are currently the only format that has been successfully used to demonstrate protection postintoxication.[141,149] Future use of antibodies against ricin intoxication may require a mixture of antibodies to both RTA and RTB to provide protection against the multiple mechanisms of intoxication. Humanizing these early protective antibodies will be essential in reducing the therapeutic agent's immunogenicity and expanding its pharmacokinetic capabilities.

Clostridium botulinum is a gram-positive, spore-forming, anaerobic bacteria that secretes neurotoxins causing botulism.[150] *C botunlinum* produce multiple serotypes of the neurotoxin BoNT, from A to G (denoted BoNT/A to BoNT/G), of which, types A, B, E, and F have been shown to cause human botulism.[136] In 2011, the Centers for Disease Control and Prevention discontinued the investigational new drug (IND) use of the pentavalent (ABCDE) botulinum vaccine, leaving a therapeutic gap in the protection of at-risk workers.[151] Although most of the treatments for botulism are based on supportive measures (eg, intensive respiratory care), passive administration of anti-BoNT immune sera has been used for treatment. A civilian working group on biodefense recommended the utilization of antisera immediately following diagnosis,[152] and treatment with equine antisera within 24 hours has demonstrated success.[153] Two therapeutic products have been derived from equine pAbs for biodefense use.[137] Well-suited to protect against the various serotypes of BoNT, these formulations were prepared, although not used, during Operation Desert Storm and Operation Desert Shield in the 1990s. The new heptavalent botulinum antitoxin (HBAT, Cangene Corp, Winnipeg, Canada) is composed primarily of Fab and $F(ab')_2$ fragments and is available from the Centers for Disease Control and Prevention under an IND protocol for naturally-acquired botulism.[137] New formulations of oligoclonal mixtures are being developed to recapitulate broad serotypic capacity, providing a more efficacious product and extended half-lives in humanized formats. Two parallel efforts, currently under development by the United States and European Union, are to develop a panel of antibodies against BoNT/A/B and /E (XOMA 3AB and AntiBotABE, respectively).[139,147,154]

Even given the rapid clearance and success of polyclonal and novel monoclonal antibody mixtures, the antibodies are effective only against the circulating toxin, and often against just a single serotype or subtype each. Once the toxin is attached to the receptor, it is internalized by receptor-mediated endocytosis, ultimately blocking neuromuscular communication. However, the administration of antitoxin may still be necessary because, even after the onset of symptoms, BoNT may remain in the bloodstream throughout infection, and clearance is essential for subsequent recovery. The oligoclonal approach to developing a cocktail against multiple serotypes is necessary for any future

TABLE 28-2

EMERGING ANTIBODY-BASED BIODEFENSE THERAPEUTICS

International Nonproprietary Name, Product Name, or Code	Targeted Agent	Targeted Antigen	Category*	Antibody Type (Isolation)	Approval, Clinical Phase, or Stage	US Patent or Reference No.
HBAT (Cangene Corp, Winnipeg, Canada)	*Clostridium botulinum* toxin (botulism)	BoNT (A–G)	A	Polyclonal Fab and $F(ab')_2$ fragments	IND treatment	(137)
XOMA 3AB (Xoma Corp, Berkeley, CA)	*C botulinum* toxin (botulism)	BoNT (A)	A	Recombinant IgG1 mAbs (human and murine origin)	Phase 1	NCT01357213
AntiBotABE (EU Consortium, 7th Framework Programme, Brussels, Belgium)	*C botulinum* toxin (botulism)	BoNT (A)	A	Recombinant IgG mAb (phage library derived)	R&D	(138–140)
RAC18	Ricin toxin from *Ricinus communis*	Ricin A chain	B	Murine mAb	R&D	(141)
c4C13	Ricin toxin from *R communis*	Ricin A chain	B	Chimeric mouse/human mAb	R&D	(142)
43RCA	Ricin toxin from *R communis*	Ricin A chain	B	scFv antibody fragment	R&D	(143)
HuMAb-154	SEB	SEB	B	Human IgG1	R&D	(144)
FL9, FL10	SEB	SEB	B	Human IgG mAbs (phage library derived)	R&D	(145)
Shigamabs cαStx1 and cαStx2v (Thallion Pharmaceuticals, Inc, Montreal, Canada)	Food- and water-borne pathogens	cαStx1 and cαStx2 Shiga toxins	B	Chimeric mouse/human mAb	Phase 2	NCT01252199

*National Institute of Allergy and Infectious Diseases category A, B, and C priority pathogens. (See https://www.niaid.nih.gov/research/emerging-infectious-diseases-pathogens for a breakdown of the classification of these biothreat categories.)

HBAT: heptavalent botulinum antitoxin; IgG: immunoglobulin G; IND: investigational new drug; mAb: monoclonal antibody; R&D: research and development; SEB: staphylococcus enterotoxin B

Data sources (as per chapter reference list): (137) Centers for Disease Control and Prevention. Investigational heptavalent botulinum antitoxin (HBAT) to replace licensed botulinum antitoxin AB and investigational botulinum antitoxin E. *MMWR Morb Mortal Wkly Rep*. 2010;59:299. (138) Avril A, Miethe S, Popoff MR, et al. Isolation of nanomolar scFvs of non-human primate origin, cross-neutralizing botulinum neurotoxins A1 and A2 by targeting their heavy chain. *BMC Biotechnol*. 2015;15:86. (139) Miethe S, Rasetti-Escargueil C, Liu Y, et al. Development of neutralizing scFv-Fc against botulinum neurotoxin A light chain from a macaque immune library. *Mabs*. 2014;6:446–459. (140) Rasetti-Escargueil C, Avril A, Chahboun S, et al. Development of human-like scFv-Fc antibodies neutralizing Botulinum toxin serotype B. *Mabs*. 2015;7:1161–1177. (141) Pratt TS, Pincus SH, Hale ML, Moreira AL, Roy CJ, Tchou-Wong KM. Oropharyngeal aspiration of ricin as a lung challenge model for evaluation of the therapeutic index of antibodies against ricin A-chain for post-exposure treatment. *Exp Lung Res*. 2007;33:459–481. (142) Guo JW, Shen BF, Feng JN, Sun YX, Yu M, Hu MR. A novel neutralizing monoclonal antibody against cell-binding polypeptide of ricin. *Hybridoma (Larchmt)*. 2005;24:263–266. (143) Pelat T, Hust M, Hale M, Lefranc MP, Dübel S, Thullier P. Isolation of a human-like antibody fragment (scFv) that neutralizes ricin biological activity. *BMC Biotechnol*. 2009;9:60. (144) Drozdowski B, Zhou Y, Kline B, et al. Generation and characterization of high affinity human monoclonal antibodies that neutralize staphylococcal enterotoxin B. *J Immune Based Ther Vaccines*. 2010;8:9. (145) Larkin EA, Stiles BG, Ulrich RG. Inhibition of toxic shock by human monoclonal antibodies against staphylococcal enterotoxin B. *PLoS One*. 2010;5:e13253.

product because rapid diagnostic determination of the toxin may not be available at the time of intoxication.

Staphylococcal enterotoxin B (SEB), one of multiple virulence factors of the gram-positive bacterium *Staphylococcus aureus*, is an extremely antigenic toxin in a family of many staphylococcal enterotoxins. Contact with naturally occurring *S aureus* can occur with these bacteria as they grow on the skin, mucosal surface, and can be found in food items. Previous proof-of-concept studies have demonstrated protection against toxic shock syndrome associated with staphylococcal enterotoxins utilizing human-derived IgG.[155] Currently, only a few mAbs targeting SEB toxins have been tested, and these used mouse models of protection. Derived from a human phage-display library, the first two mAbs, FL9 and FL10, demonstrated protection when premixed with the toxin.[145] HuMAb-154, a human antibody derived from hybridoma technology, demonstrated partial protection and delayed time to death when administered following increasing lethal doses of intraperitoneally administered SEB.[144] The human origin of these three antibodies supplies the advantage of reduced immunogenicity when intended for human use. However, these antibodies have yet to be used in a primate model or against aerosol exposure, experiments necessary to establish efficacy of the therapeutic potential against SEB for biodefense.

One of the main issues concerning toxin therapy using mAbs is treatment beyond 24 hours postexposure. Antitoxin antibodies are most effective when given early after exposure, and these timelines can be restrictive given the time it takes to detect and identify the toxin of interest. Although anti-ricin antibodies, when administered effectively, clear all toxins, antitoxin antibodies inhibit the activity of the toxin only, and not the pathogen itself (ie, neutralizing BoNT versus treating *C botulinum*). Thus, in the case of an infection, a treatment plan should be developed to eliminate both the toxin and the pathogen responsible for its production.

Antibacterial Monoclonal Antibodies

Many bacterial agents produce toxins. In addition to using mAbs against these bacterial toxins, mAb prophylaxis and therapy has targeted components on the surface of the bacterium, as with anthrax.[156] As early as 1890, antibacterial serum therapy proved successful, with the application of immune serum against diphtheria and tetanus developed by Emil von Behring, a German military doctor, who received a Nobel Prize for the initial development of pAbs.[132] With the germ theory growing more widely accepted throughout the 1900s, the first antibiotics were concurrently developed and often overshadowed the use of serum and antibody therapy. However, an increase in bacterial resistance has been observed across all existing antibiotic classes highlighting the need for the identification of new therapeutic options.[157] The early success of antibody-based therapies starting with polyclonal formulations and now more recent mAbs may constitute a therapeutic class capable to fill therapeutic gaps or even augment existing current therapies. Given the success of mAbs against cancer inflammatory and autoimmune diseases, it is surprising that more anti-infective mAb treatments have failed to make it to market. The primary factor inhibiting the progress of antibacterial mAbs is their lack of efficacy in animal studies and clinical testing, despite promising preclinical data.[156] (See Table 28-3[157–163] for more details.)

Anthrax, from *B anthracis*, is an agent that has been previously weaponized by state-sponsors and more recently used in an intentional dissemination of spores in the United States in 2001.[164] Early development of human antibody-based therapeutics against anthrax began after earlier antibodies of animal origin increased the therapeutic window of the disease and also shortened the duration of antibiotic treatment. Anthrax-specific antibody-based therapeutics act by a variety of mechanisms, either by targeting the capsule or by neutralizing the toxins that treat or augment therapy.[156] These antibodies represent the most advanced biodefense-related therapeutics, with the only two biodefense class specific mAbs, approved, and several others advancing through preclinical and clinical trials. These represent some of the most widely studied antibodies in the biodefense arena, with multiple mechanisms of action identified. Raxibacumab and Anthim (Elusys Therapeutics, Pine Brook, NJ) act by inhibiting PA-receptor interactions. Murine-derived antibodies 7.5G and 48.3 inhibit PA cleavage by furin, but thus far they have only been tested in murine models.[165,166] Thravixa (previously AVP-21D9; Emergent BioSolutions), currently in phase I clinical trial, inhibits PA heptamerization. Although no mAbs that directly inhibit the PA-LF/EF complex interaction are currently undergoing clinical evaluation, the potential for therapies has been demonstrated by human mAbs IZNLF, SS87, Fab A8, and 2LF, as well as the cross-reactive mAb H10. Other mechanisms have been shown by the chimeric chimpanzee/human mAbs LF10E and LF11H, which act by inhibiting endocytosis and translocation. However, LF10E and LF11H have only been tested in rodent models. Within all of these second generation therapeutics to anthrax, the specific mechanisms are expected to increase the therapeutic window, decrease length of treatment and assist in overcoming antibiotic resistance.

TABLE 28-3

ADVANCEMENTS OF TREATMENTS FOR BIODEFENSE BACTERIAL AGENTS UTILIZING ANTIBODY-BASED THERAPEUTICS

International Nonproprietary Name, Product Name, or Code	Targeted Agent	Targeted Antigen	Category*	Antibody Type (Isolation)	Approval, Clinical Phase, or Stage	US Patent or Reference No.
Raxibacumab/ABthrax (GlaxoSmithKline, Brentford, UK)	*Bacillus anthracis* (anthrax)	Anti-PA	A	Recombinant IgG1 mAb (naïve library)	Approved (2012)	601351
Valortim (Bristol-Myers Squibb, New York, NY)/MAb-1303	*B anthracis* (anthrax)	Anti-PA	A	Human mAb (transgenic mice)	Phase 1	7456264
Anthim Elusys Therapeutics (Elusys Therapeutics, Pine Brook, NJ)/ETI-204	*B anthracis* (anthrax)	Anti-PA	A	Chimeric deimmunized mAb (murine origin)	Phase 1	7446182
Thravixa (Emergent BioSolutions, Rockville, MD)/AVP-21D9	*B anthracis* (anthrax)	Anti-PA	A	Human mAb (hybridoma)	Phase 1	7438909, 7442373
Anthriving (Emergent BioSolutions)/AIG	*B anthracis* (anthrax)	Anti-PA	A	Polyclonal antibody from AVA human plasma	Phase 1/2	N/A
mAb 7.3 (plague)	*Yersinia pestis*	LcrV	A	Murine mAb (hybridoma)	R&D	(157)
m252/m252/m254 (plague)	*Y pestis*	F1/LcrV	A	Recombinant human mAb (naïve library)	R&D	(158)
mAb 3	*Francisella tularensis* (tularemia)	LPS	A	Murine IgG2a	R&D	(159)
6B3	*Brucella* species (brucellosis)	*B melitensis* LPS	B	Murine IgG3	R&D	(160)
2C8	*Brucella* species (brucellosis)	*B abortus* LPS	B	Murine IgG3	R&D	(160)
Ps6F6	*Burkholderia pseudomallei* (melioidosis)	Exopolysaccharide	B	Murine IgG3	R&D	(161)
1G2-1D3	*B mallei* (glanders)	LPS	B	Murine IgG2a	R&D	(162, 163)

*National Institute of Allergy and Infectious Diseases category A, B, and C priority pathogens.

AIG: anthrax immune globulin; AVA: Anthrax Vaccine Absorbed; IgG: immunoglobulin G; LPS: lipopolysaccharide; mAb: monoclonal antibody; N/A: not applicable; PA: protective antigen; R&D: research and development; IgG: immunoglobulin G

Data sources (as per chapter reference list): (157) Hughes JM. Preserving the lifesaving power of antimicrobial agents. *JAMA*. 2011;305:1027–1028. (158) Hill J, Copse C, Leary S, Stagg AJ, Williamson ED, Titball RW. Synergistic protection of mice against plague with monoclonal antibodies specific for the F1 and V antigens of *Yersinia pestis*. *Infect Immun*. 2003;71:2234–2238. (159) Xiao X, Zhu Z, Dankmeyer JL, et al. Human anti-plague monoclonal antibodies protect mice from *Yersinia pestis* in a bubonic plague model. *PLoS One*. 2010;5:e13047. (160) Lu Z, Roche MI, Hui JH, et al. Generation and characterization of hybridoma antibodies for immunotherapy of tularemia. *Immunol Lett*. 2007;112:92–103. (161) Laurent TC, Mertens P, Dierick JF, Letesson JJ, Lambert C, De Bolle X. Functional, molecular and structural characterisation of five anti-Brucella LPS mAb. *Mol Immunol*. 2004;40:1237–1247. (162) Bottex C, Gauthier YP, Hagen RM, et al. Attempted passive prophylaxis with a monoclonal anti-*Burkholderia pseudomallei* exopolysaccharide antibody in a murine model of melioidosis. *Immunopharmacol Immunotoxicol*. 2005;27:565–583. (163) Trevino SR, Permenter AR, England MJ, et al. Monoclonal antibodies passively protect BALB/c mice against *Burkholderia mallei* aerosol challenge. *Infect Immun*. 2006;74:1958–1961.

Bubonic, septicemic, and pneumonic plague are all caused by the gram-negative bacterium *Y pestis*. *Y pestis* contains two dominant targets for antibody-based therapeutics: (1) the surface polymer F1 and (2) the surface protein LcrV (as previously described in Plague, Chapter 10 in this volume). Early proof-of-concept studies using the murine anti-LcrV mAb 7.3 demonstrated complete protection against an aerosolized *Y pestis* challenge.[158] Other murine antibodies demonstrated similar protection, with mAb 7.3 and F1-04-A-G1 providing protection when administered as an aerosol cocktail prior to aerosolized challenge.[167] Naïve libraries have been used to produce the recombinant mAbs m252, m253, and m254. When used in a cocktail, these antibodies demonstrate synergistic protection, but they have been tested in murine models only and in the absence of antibiotics.[159] Because antibiotics are generally the first line of treatment, an understanding of the combined efficacy of antibody therapies and antibiotics is currently lacking in the antibody-based treatment of plague.

F tularensis is extremely virulent and difficult to identify with serological tests. Although *F tularensis* is susceptible to current antibiotics, an effective antibody-based therapeutic may be necessary because the various strains use different virulence mechanisms. Although these differences may explain the difficulty in obtaining an efficacious therapeutic, they also highlight the unique nature of each strain, which could be treated individually by using specific antibodies. The potential for effective antibody therapy against *F tularensis* in an aerosolized challenge model was first demonstrated by serum transfer of *F tularensis* LVS, which increased the mean time to death against the SchuS4 challenge strain.[168] The current antibody-based therapeutic options for *F tularensis* are of murine origin and have only been tested in rodent models.[160,169] Additional research and development will be needed in these areas to advance an effective biologic for clinical application.

The most successful utilization of antibacterial mAbs to date targeted the toxins of these organisms. These mAbs often possess extremely high affinity to their toxin targets, binding to and clearing the damaging toxins while allowing time for the host's immune response to clear the underlying bacterial infection. In contrast to toxin-exclusive neutralization, clearance mechanisms play a critical role in the therapeutic efficacy of these antibodies. In addition to the Fc-mediated clearance, it has been shown that antibodies can paradoxically increase the cytotoxic effects of these agents, as was seen in the enhancement of anthrax lethal toxin.[170] This complex interaction between the requirement for antibody-mediated clearance and the potential for negative antibody-dependent enhancement has also been recently reported for virus-specific antibody therapeutics.[171]

Antiviral Monoclonal Antibodies

Historically, vaccination has been the primary means of providing any medical countermeasure against viruses with bioterrorism agent potential. Following the eradication of smallpox, outbreaks of biodefense-related viruses in populations, with the exception of yellow fever, have been relatively infrequent. Several of these agents remain poorly understood due to their sporadic occurrence or orphan nature and limitations in resources or facilities capable of researching these viral diseases. The high mutation rate and poor understanding of many of these viruses account for the therapeutic gaps in protection, while the high infectivity and mortality make these an optimal bioweapon. For many viruses considered to have potential for use as biological weapons, including viral hemorrhagic fevers (VHFs) (eg, Ebola virus, Sudan virus, and Marburg virus) and *Togaviridae* encephalitides (eg, VEEV, eastern (EEEV), and western (WEEV) equine encephalitis viruses), only supportive therapy exists following exposure. Ribavirin, a nonimmunosuppressive nucleoside-analogue with broadly protective antiviral properties, has demonstrated protection only against the VHF Lassa virus and is available only for compassionate use under an IND application.[172] Additional studies have indicated that the use of ribavirin would be effective against other arenaviruses as well as Bunyaviruses (see Alphaviruses, Chapter 20 in this volume). Beyond ribavirin, few other drug options exist, leaving only supportive care in many cases.

The successful application of therapeutic antibodies has been demonstrated previously. The National Institute of Allergy and Infectious Diseases (NIAID) categorizes biodefense agents based on several factors including, but not limited to, the agent's pathogenesis, dissemination, available treatments, as well as the agent's mortality. Category A agents are considered the most dangerous, based on ease of dissemination, high mortality, public health impact, and absence of available therapeutics, with Categories B and C following to a lesser extent in one or all of these areas. This chapter has focused primarily on Categories A and B agents; however, two category C agents, Hendra virus and Nipah virus, have well-characterized therapeutic mAbs for postexposure treatment. (See Table 28-4[173–183] for more details.) The human mAb m102.4 demonstrated protection against Nipah in the ferret model[184] and Hendra in a nonhuman primate (NHP) model[183] as a postexposure therapeutic as late as 7 to 8 days after infection.

TABLE 28-4

ADVANCEMENTS OF TREATMENTS APPLIED ON BIODEFENSE VIRAL AGENTS UTILIZING ANTIBODY-BASED THERAPEUTICS

International Nonproprietary Name, Product Name, or Code	Targeted Agent	Targeted Antigen	Category*	Antibody Type (Isolation)	Approval, Clinical Phase, or Stage	Patent, Trial Number, or Reference No.
ZMapp (13C6, 2G4, 4G7)	Filovirus (Ebola)	Ebola Zaire GP	A	Cocktail of three recombinant human mAbs	Phase 1/2, utilized EUA	NCT02389192, NCT02363322 (173)
ZMab (1H3, 2G4, 4G7)	Filovirus (Ebola)	Ebola Zaire GP	A	Cocktail of three recombinant human mAbs	Utilized EUA	(174)
MB-003 (13C6, 13F6, 6D8)	Filovirus (Ebola)	Ebola Zaire GP	A	Cocktail of three recombinant human mAbs	Utilized EUA	(175)
8AH8AL	Variola major (smallpox)	B5	A	Chimeric chimpanzee/human mAb	R&D	(176)
6C	Variola major (smallpox)	A33	A	Chimeric chimpanzee/human mAb	R&D	(177)
hV26 / h101	Variola major (smallpox)	H3/B5	A	Human mAbs (transgenic mice)	R&D	(178)
hB5RmAb	Variola major (smallpox)	B5	A	Human mAb (derived from rat)	R&D	USP 7811568
Vaccinia immune globulin	Variola major (smallpox)	Whole antigen	A	16.5% IgG from vaccinia virus individuals	Approved for vaccine SE	(179)
Hu1A3B-7	VEE	E2 GP	B	Humanized mAb IgG1 (derived from mouse)	R&D	(180)
Hu1A4A-IgG1-2A	VEE	E2 GP	B	Humanized mAb IgG1 (derived from mouse)	R&D	(181)
ToR67-3B4	VEE	E1 GP	B	scFv-Fc fusion protein (NHP phage display)	R&D	(182)
m102.4	Nipah / Hendra	GP G	C	Recombinant IgG1 mAb	Preclinical development	(183)

*National Institute of Allergy and Infectious Diseases category A, B, and C priority pathogens.
EUA: Food and Drug Administration Emergency Use Authorization; GP: glycoprotein; mAb: monoclonal antibody; NHP: nonhuman primate; R&D: research and development; SE: side effects; USP: US Pharmacopeia; VEE: Venezuelan equine encephalitis
Data sources (as per chapter reference list): (173) Qiu X, Wong G2, Audet J, et al. Reversion of advanced Ebola virus disease in nonhuman primates with ZMapp. *Nature*. 2014;514:47–53. (174) Qiu X, Audet J, Wong G, et al. Sustained protection against Ebola virus infection following treatment of infected nonhuman primates with ZMAb. *Sci Rep*. 2013;3:3365. (175) Pettitt J, Zeitlin L, Kim do H, et al. Therapeutic intervention of Ebola virus infection in rhesus macaques with the MB-003 monoclonal antibody cocktail. *Sci Transl Med*. 2013;5:199ra113. (176) Chen Z, Earl P, Americo J, et al. Chimpanzee/human mAbs to vaccinia virus B5 protein neutralize vaccinia and smallpox viruses and protect mice against vaccinia virus. *Proc Natl Acad Sci U S A*. 2006;103:1882–1887. (177) Chen Z, Earl P, Americo J, et al. Characterization of chimpanzee/human monoclonal antibodies to vaccinia virus A33 glycoprotein and its variola virus homolog in vitro and in a vaccinia virus mouse protection model. *J Virol*. 2007;81:8989–8995. (178) McCausland MM, Benhnia MR, Crickard L, et al. Combination therapy of vaccinia virus infection with human anti-H3 and anti-B5 monoclonal antibodies in a small animal model. *Antivir Ther*. 2010;15:661–675. (179) Hopkins RJ, Lane JM. Clinical efficacy of intramuscular vaccinia immune globulin: a literature review. *Clin Infect Dis*. 2004;39:819–826. (180) Goodchild SA, O'Brien LM, Steven J, et al. A humanised murine monoclonal antibody with broad serogroup specificity protects mice from challenge with Venezuelan equine encephalitis virus. *Antiviral Res*. 2011;90:1–8. (181) Hu WG, Phelps AL, Jager S, et al. A recombinant humanized monoclonal antibody completely protects mice against lethal challenge with Venezuelan equine encephalitis virus. *Vaccine*. 2010;28:5558–5564. (182) Rulker T, Voss L, Thullier P, et al. Isolation and characterisation of a human-like antibody fragment (scFv) that inactivates VEEV in vitro and in vivo. *PLoS One*. 2012;7:e37242. (183) Geisbert TW, Mire CE2, Geisbert JB, et al. Therapeutic treatment of Nipah virus infection in nonhuman primates with a neutralizing human monoclonal antibody. *Sci Transl Med*. 2014;6:242ra82.

Before the development of mAb-based therapeutics, passive antibody therapy had been the only option for treating VHFs, but these efforts yielded mixed results.[185] Despite previous successful results, concerns about the transmissibility of blood-borne pathogens from donor sera to recipients remain. In many cases, the diagnostic screening capabilities needed to confirm that a sample is pathogen-free before administration within the therapeutic window are not present. However, passive immunotherapy has provided the initial necessary evidence that therapeutic antibodies can be an effective preexposure or postexposure therapeutic.[185]

Despite the promise shown by passive immunotherapy, early negative experimental evidence limited interest in the application of antibody-based therapeutics against VHFs, specifically the Filoviruses. Previous passive transfer studies against Lassa virus demonstrated effectiveness if given early in the course of infection.[186] During the 1995 Kikwit Ebola outbreak, crude blood transfusions were used as an immunotherapy, resulting in a death rate of one in eight, in comparison to an 80% mortality rate for those untreated.[187] Studies using hyperimmunized equine serum against Ebola virus administered to macaques demonstrated delayed time to death but no change in the survival rate.[188] The first attempt at protecting against Ebola virus used KZ52, a human antiglycoprotein mAb.[189] KZ52 effectively neutralized Ebola virus in plaque assays and, when passively administered, protected guinea pigs,[190] but it failed to protect or affect disease progression when given to NHPs.[191]

One explanation for the failures of passive immunization of pAbs or single mAbs to protect against Filoviruses is that this therapy controls the viral burden initially, but once depleted, the virus overwhelms the system. However, in 2012 passively transferred species-matched pAbs were found to provide complete protection in an NHP model, demonstrating the capability of antibody-based therapies against Filoviruses.[192] Recent oligoclonal, or cocktail, mixtures of antibodies that target multiple epitopes of the virus—such as MB-003,[175] ZMAb,[174] and ZMapp[173]—have demonstrated protection in infected NHPs. Furthermore, ZMapp was administered under emergency use authorization to two healthcare providers infected with Ebola during the 2014 West Africa outbreak who subsequently survived the infection.[3] Similar to vaccines that provide humoral immunity, such as VEEV replicon particles,[193] ZMAb provided complete sustained protection 10 weeks posttreatment upon re-challenge in NHPs.[174] These studies, taken together with published data specific to the correlates of Filovirus protection,[194] provide evidence of the ability of antibody therapy to provide protection until the host's humoral immune system initiates a response.

Historically, smallpox is one of the most concerning of all biothreat agents. Even with a successful vaccination program, smallpox remains a potential biological weapon because of the large nonvaccinated population. Other than vaccination, the only approved therapeutic is vaccinia immune globulin, a pAb extracted from vaccinated humans. Despite its limited potency against the disease,[179,195] this product is approved to reduce the potential side effects of the vaccine and has been shown to reduce morbidity and mortality associated with smallpox.[179] Several products have been developed using animal-based isolation techniques, although none are in clinical trials. Given the restricted access to the smallpox virus, new antibody-based therapeutics have been compared with vaccinia immune globulin using the vaccine strain for challenge. Several products have emerged in recent years from phage-display technology using chimpanzees,[176,177] transgenic mAbs,[178] and humanized rat mAbs[196]; mAbs developed against smallpox have been isolated from either vaccinated or infected animals. The humanized mAb hB5RmAb, whose parental antibody was isolated from a rat, and 8AH8AL, a chimeric chimpanzee/human recombinant antibody (rAb) derived from phage display, are both directed against the B5 surface protein of the extracellular enveloped virions.[176,196] Additionally, two human mAbs, hV26 and h101, were isolated from transgenic mice and bind to the H3 protein found at the surface of mature virus and to the B5 protein, respectively.[178] All of these antibodies elicited protection in various mouse models; however, none have been tested in NHP models of disease, as recommended by the World Health Organization.

Several viruses of the Alphavirus genus cause encephalitis, and of these, VEEV and EEEV are classified as category B select agents. Early animal studies using passive transfer of neutralizing antisera and mAbs demonstrated this therapy's protection against Alphaviruses.[197–199] Early proof-of-concept studies have shown human constructs and recombinant mAbs to be successful in providing protection in mice.[181,200,201] The humanized mAb Hu1A3B-7 (IgG1 isotype) binds the E2 glycoprotein and is broadly specific to VEEV subtypes, neutralizing type IAB (Trinidad donkey or TrD), type II (Fe37c), and type IIIA (Mucambo BeAn8) in vitro. Hu1A3B-7 administered intraperitoneally 24 hours postchallenge provided complete protection against subcutaneous challenge of 100 times the median lethal dose (LD_{50}) of VEEV TrD, as well as 90% protection when challenged against 100 LD_{50} by aerosol.[200] Hy4 is a humanized antibody that binds to the VEEV E2

protein. When administered intraperitoneally 1 or 24 hours postchallenge with VEEV TrD, just 10 μg elicited 90% and 75% protection. Administration of 500 μg of Hy4 24 hours prechallenge provided 80% protection against intranasal challenge of 1,350 plaque-forming units.[201] The mAb Hu1A4AIgG1-2A, humanized from murine 1A4A, binds to the E2 glycoprotein of VEEV with high affinity. The Hu1A4AIgGIgG1-2A provided both prophylactic and therapeutic protection against subcutaneous administration of VEEV TrD.[181] The recently developed ToR67-3B4, an NHP phage-display derived scFv-Fc fusion antibody directed to the E1 protein, represents one example of the next generation of constructs providing protection against Alphaviruses.[182] This antibody provided 83% protection against an aerosol challenge of VEEV TrD, with limited protection at later times in the mouse model.[182] The antibodies that protected against VEEV were only tested in mice. None of the above antibodies have been tested in larger models; any potential therapeutic for biodefense would need to be used in models currently under development.[202]

Unlike other Department of Defense biothreat agents, Alphaviruses have a neuroinvasive component, which limits the effectiveness of antibody-based therapeutics due to their inability to cross the blood–brain barrier (BBB). Although active infection can inhibit the BBB's ability to filter, allowing some immune therapeutics to pass through late during infection, new therapeutic designs with the ability to cross the BBB would facilitate treatments. Developers of the next generation of Alphavirus-based antibodies should endeavor to design antibodies with a capability to cross the BBB. One possible format for these therapeutics would be bispecific antibodies (see Figure 28-3). Several antibodies have been developed for diseases and conditions unrelated to biodefense that could serve as a model for the development of BBB-crossing therapeutic mAbs against Alphaviruses.[203] Other antibody formats developed outside of biodefense include complete trifunctional chimeric IgGs and the scFv-scFv constructs.[204] While a broadly reactive mAb is ideal, the high mutation frequency of Alphaviruses and other RNA viruses raises concern about the potential emergence of resistant strains.[147] A monoclonal therapeutic will most likely require an oligoclonal product, or cocktail of several antibodies, each broadly reactive and capable individually of neutralizing the virus.

Any successful viral infection requires viral particles to be released into extracellular space; however, with some biodefense-related viruses, such as variola major, that require just one inhaled particle-forming unit to initiate an infection, the utility of circulating antibodies becomes limited.[205] Viral latency has been one of the major challenges in developing effective antibody-based therapeutics against HIV.[206] Furthermore, as demonstrated by the brief protection windows described above, many biodefense-related viral pathogens require rapid identification and antibody administration within the first 48 to 72 hours to be effective. This is typically the case for Alphaviruses that cross the BBB and Filoviruses that rapidly overwhelm the immune system with high viral load.

In addition to rapid diagnosis, having a greater understanding of biodefense agents and their pathogenesis in host model systems will greatly aid in the ability to more quickly identify and develop therapeutics. For example, the identification of Alphavirus glycoprotein glycosylation sites and successful production of recombinant glycoproteins would allow for rapid screening of target antibodies. Similarly, the development of an animal model for Crimean-Congo hemorrhagic fever would supply an in vivo model system to test therapeutic efficacy. These are just two examples of basic tools and knowledge that could significantly enhance the productivity and efficacy of vaccine and therapeutic development efforts. To achieve the greatest success, future work should therefore focus on the development of appropriate reagent material and model development in parallel with the programmatic development of therapeutic and vaccine candidates.

SUMMARY

Vaccines and antibody-based therapeutics are some of the greatest achievements in global health improvement in previous centuries. Despite these advances, biodefense vaccines and treatments remain scarce, and there is a great need to define future requirements specific to biodefense vaccines and antibodies. Enormous advances have been made in the fields of vaccines and antibody-based medical countermeasures, and many creative strategies have been developed that may address the current needs; however, the barriers between an idea or concept and a product are vast, and costs to develop one product can surmount $100 million.

The first challenges in the future development of vaccine or immunotherapy medical countermeasures will be how to prioritize the funding for an ever-growing pipeline of products and whether to develop vaccines or antibodies (or a combination of both). Within the limited funding environment of infectious diseases and toxins, focus should be agent-specific for the development of specific vaccines and antibody-based therapeutics. One option would be to focus vaccine

development primarily on communicable diseases, for which the threat of epidemic outbreaks is a primary concern. Then, noncommunicable diseases and toxins, or those diseases that are poorly transmitted, could be addressed by development of antibody-based therapeutics. Alternative approaches could then be used to augment the initial round of medical countermeasures.

New vaccine and therapeutic development should not only be aligned with the relative ease in obtaining many of the more historical biological agents (eg, ricin or anthrax) as a determining factor, but also should be aligned to the categorization of the agents and availability or absence of availability of effective vaccines and/or therapeutics for the higher category agents. The Amerithrax anthrax attacks highlight the panic and fear that can quickly disrupt public, commercial, and governmental activities with localized instances of infection. This public fear perception is the principal reason why Filovirus infections, specifically Ebola virus, attract so much attention in contrast to other infectious diseases that kill far more people annually from ongoing epidemic outbreaks (eg, influenza virus). In the United States, a new strategy using a "whole-government" approach has been implemented by the National Interagency Confederation for Biological Research to coordinate efforts for the development of medical countermeasures.[207] Internationally, NATO (North Atlantic Treaty Organization) panels, cooperative agreements, and basic science partnerships are also being used to reduce the overall cost and impact of incorporating more novel means for developing countermeasures on tighter budgets. Involvement in these cooperative strategies should be leveraged at the interagency and international level as a means of cost reduction, as well as a diversification of expertise as the community searches for the next generation of medical countermeasures.

Biodefense vaccines may also face several more general issues of approval and licensure, much like common vaccines, but with the added requirements of approval under the Animal Rule because many of these diseases do not exist for an adequate Phase II/III study. Vaccine approval, even in the military, can be challenging, as illustrated by the anthrax vaccine campaign that was interrupted by the Department of Defense in 1999.[208] Vaccine safety standards have become more stringent over the last few decades, and biodefense vaccines must be held to these standards. Whereas confidence in vaccine efficacy has also improved, the task of ensuring vaccine safety can be daunting, as exemplified in spring 2010 by the increased risk of narcolepsy and catalepsy observed in patients in several countries after H1N1 vaccination.[209] Another concept that may need to be integrated into biodefense vaccines is individualized medicine. Next-generation vaccines may be targeted to specific subpopulations according to their HLA genotypes or the capacity of their immune system to mount an appropriate immune response.

The next generation of antibody development should focus on the mechanism of how antibodies enter into or influence a cellular environment. For example, "transbodies" are cell-permeable antibodies made through conjugation of an antibody to a ligand to facilitate entry of the antibody into the cell or to inhibit a specific function, as with immunotoxins. Conversely, "intrabodies" are antibodies developed to achieve intracellular expression using the application of recombinant DNA technology.[210]

Even with these novel means of antibody delivery and action, target identification remains one of the largest challenges in developing the next effective antibody therapeutics. Secondarily, antigen-binding specificity and access are other points to consider because antibodies are highly specific to the target, and corresponding antigens from different species, such as NHPs or rodents, are dependent on the antigen alignment between species. In many cases, the critical epitopes targeted by vaccines and therapeutics are conserved and additional mitigation has been achieved by the use of oligoclonals, or cocktails of antibodies. These critical epitopes are of particular concern for both vaccines and therapeutic antibodies because biodefense-related treatments often require special use of the FDA Animal Rule for advancement through clinical trials.

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Chapter 29

AEROBIOLOGY: HISTORY, DEVELOPMENT, AND PROGRAMS

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INTRODUCTION

CONCEPTUAL BASIS OF AEROBIOLOGY IN INFECTIOUS DISEASE

OFFENSIVE BIOLOGICAL DEVELOPMENT AND CLINICAL APPLICATIONS IN THE UNITED STATES
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BIOLOGICAL AEROSOL EXPOSURE SYSTEMS
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- **Sampling**
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- **Parameters Impacting Aerosol Dosimetry**

SUMMARY

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INTRODUCTION

The concept of using inhaled infectious particles as biological weapons is not new. The significance of this route was first appreciated and truly understood in the early 20th century, although the concept of infection by inhalation has been intermittently influenced by the study of infectious disease epidemiology. The question of whether diseases are "air-caused" has had, in past centuries, ardent believers and equally passionate cynics. Historically, for example, the prevailing theory was that all infections originated from "miasma," or contaminated air. The cyclic nature of disease transmitted by aerosol among people living in groups is described in basic terms in ancient preserved artifacts. The Smith papyrus, dating from 1600 BCE and held in the Field Museum of Natural History in Chicago, describes prayers recited to gods of disease to purify the "winds" of the "pestilence of the time."[1] Epidemics were thought to be transmitted by aerosol even in the early days of medical science. Around 400 BCE, Hippocrates dictated that "airs, waters, and places" directly influenced the health of people, and he used the knowledge of seasonal change to guide diagnosis of differing ailments. In the Middle Ages, few pathogens impacted understanding of epidemic spread of disease as *Yersinia pestis*, the causative agent of the "black death." At-risk populations eventually learned that the only defense against infection and death was to avoid contact with victims dying or dead from the bacterial disease. Pneumonic plague, the corollary form of infection from an infected host, is now recognized to transmit from expectoration of respiratory droplets. People may have unwittingly avoided respiratory exposure to aerosolized *Y pestis* by avoiding contact with infected hosts and thereby not contracting the most feared (and deadly) form of the bacterial disease.

Advancements in the field of chemistry in the 19th century gave rise to the concept of miasmic theory of disease. Sir Edwin Chadwick (1850) in Britain advanced the public health practices associated with the avoidance of the malodorous vapors to preserve the overall health of at-risk populations. The concept of spontaneous generation of disease-causing agents in vapors, however, was countered and ultimately refuted by Louis Pasteur (1860) during the same era. Pasteur demonstrated the presence of living organisms that was the root cause of fermentation and decomposition. His work in this area was instrumental in the understanding that infection could only appear miasmic if airborne microorganisms were present. By the end of the 19th century, most communicable bacterial pathogens had been identified, and there were only a few effective airborne agents. At roughly the same time, seminal work in vector-borne disease, including demonstration of parasitic disease cycles for malaria and filariasis, further improved public health measures and reduced disease burden. The concept that the majority of disease agents affecting large numbers of people were food- or water-borne greatly minimized aerosol transmission as an important pathway of infection.

In the early 20th century, it was also shown that respiratory droplets from diseased individuals, never traveling more than an arm's length from the infected person, could readily transmit disease. The theory of large-droplet infection, coupled with the recognition that arthropods were vectors for disease, nearly negated the respiratory route of infection from consideration in natural endemic spread of disease.[2] It was not until the great influenza pandemic of 1917–1918 that airborne disease transmission was again considered a medically important infection route. The notion that near-instant dehydration takes place in the environment once numerous particles are expelled from an infected host, causing submicron infectious biological aerosols to "float" for hours, solidified the concept of ecological transport from an infected host to an otherwise naïve host and ultimately successful disease transmission. A more modern understanding of airborne contagion also dictated that the probability and rate of disease transmission through air differs from, for example, a contaminated well.[3] Although the number and tempo of infections from a contaminated well are horizontal, arithmetic, and limited to the number of interactions with a single source, airborne disease transmission is truly a vertical and geometric process and is not limited to interactions with one infected source, but rather the general vicinity of one or many infection sources.[4] The indoor environment that now comprises most of the modern world amplifies probability of vertical transmission from airborne pathogens.

There is a sharp distinction between naturally communicable airborne disease and those that are artificially induced through human-made biological aerosols. Modern military and ancillary industrial development activities, primarily associated with offensive biological weapon development in the 20th century, exploited the characteristics of aerosols that would promote maximum potential impact upon enemies.[5] This was primarily achieved by modern and sophisticated manipulation of the particulars, such as particle size distribution and environmental dehydration, to assure successful delivery to the respiratory system of the target host population. An early scientific

concept in the process of designing and producing biological aerosols as modern weapons was the research and understanding of naturally occurring airborne disease. A basic, empirically derived understanding of natural epidemics from human source generators (respiratory expectoration) and indirect sources (eg, fomites on bed sheets) was essential to better appreciate important environmental and physiochemical factors when designing biological aerosols. It was soon recognized that airborne infection, when left up to the natural transmission process, was an overwhelmingly variable process influenced by a number of intrinsic and extrinsic factors, many of which cannot be readily controlled. The process of natural spread of disease by the aerosol route was described in detail in studies predating World War II; comprehensive descriptions were first published in the eminent text, *Airborne Contagion and Air Hygiene,* by WF Wells.[1] Many of the early tenets of infection from droplet nuclei are presented in this work, with descriptions of experiments that demonstrate the most basic mechanisms dictating infection from an airborne microbial source.

CONCEPTUAL BASIS OF AEROBIOLOGY IN INFECTIOUS DISEASE

The basic mechanism for transmitting airborne disease is by droplet nuclei. Droplet nuclei have been described as small, air-suspended residues arising from the evaporation of droplets emanating from the mouth and nose. These nuclei-containing infectious microbes (bacteria or viruses) or toxic components collectively comprise biological aerosols that are medically important. Such aerosols are readily produced artificially by spraying or atomizing wet or dried preparations of microorganisms or toxins.

There are many experimental uses of aerosols, but those used for respiratory disease studies are especially important.[3] The study of disease pathogenesis in animal models can be more meaningful if subjects are infected by the same route that occurs naturally in humans. In contrast to intratracheal or intranasal instillation, infectious challenge with aerosol particulates greatly increases natural dispersion in the respiratory system and is consistent with "natural" aerosol infection. Dosage, aerosol particle size, age, environmental temperature, and humidity can all be measured, controlled, and analyzed to some extent.[6] Moreover, the interplay of these features can be studied in the context of microbial viability and resulting virulence.

There are disadvantages, however, that are inherent in aerobiological experimentation. The significance of aerosol age on airborne organism virulence is not fully known. Finally, respiratory doses are difficult to reliably calculate because the degree of lung retention of inhaled aerosol particles, while predictable, usually is not measured.[7] Experiments involving aerosol challenge of animals include determining the host species' susceptibilities, estimating or establishing dose-response curves, evaluating the effect of therapy or stress, and testing the efficacy of experimental vaccines.

These early studies made clear that measuring and controlling as many of the variables as possible associated with stability, viability, and corresponding infectivity of virulent biological aerosols was required for the first biological weapons produced using modern technological methods. Rapid industrialization of the microbiological and evaluation aspects of developing biological weapons was pursued by the militaries of world powers at the time, which ushered in an era of aerobiological research that was performed on a grand scale.

Military programs throughout the mid-20th century engaged in researching and developing biological weapons selected aerosol as the predominant modality and route of battlefield delivery to the enemy. A historic brain trust, comprised of the personnel and physical resources capable in this scientific area, was developed among the superpowers to support this effort. The extensive network that developed was uniquely qualified to harness and perfect the biological, physiochemical, and logistical characteristics preferential to aerosol stability and survival for industrial production and eventual delivery in munitions.

OFFENSIVE BIOLOGICAL DEVELOPMENT AND CLINICAL APPLICATIONS IN THE UNITED STATES

Camp Detrick, Black Maria, and the US Army Medical Unit (1954–1970)

In 1941, the Secretary of War asked the National Academy of Sciences (NAS) to review the risk of biological warfare if the United States were to become engaged in World War II. The War Bureau of Consultants from the NAS advised the Department of Defense to prepare for biological warfare and to provide the resources for both defensive and offensive capabilities. In the spring of 1942, the Army determined that the first US Army biological warfare laboratories would be located at Camp Detrick (Army Air National Guard [ANG] Airfield) in Frederick,

Maryland.[8] Before the offensive and defensive efforts were pursued at Camp Detrick, the Safety Division made great strides in developing capabilities for biocontainment, decontamination, and sterilization of hazardous disease agents. Biological weapon production and testing facilities were initially built at Camp Detrick for the purpose of producing anthrax and botulinum neurotoxin for weapons. The first research facility was located in the ANG hangar, which was modified to include laboratories. A seven-story pilot plant facility was built in 1943 to test fermenters to find the most optimal configuration for culturing large amounts of organisms such as *B anthracis*. A free-standing building was constructed to house this operation; it was covered with the most impervious material available at the time (tar paper), which gave the structure the appearance of an ominous black box and invoked the moniker "Black Maria." This facility was later dismantled to make way for larger, more modern buildings. The US expanded its offensive biological warfare efforts to include production and storage facilities at the Pine Bluff Arsenal in Arkansas, and in Terre Haute, Indiana, during the Cold War in the wake of World War II. The capability at Fort Detrick and ancillary production facilities throughout the United States provided the source material for initial efforts in preparing and packaging biological agents capable of being dispersed as aerosols via munitions. The microbiological expertise and industrial-sized production capabilities during these early efforts were essential for biological stability, which was required for continual production of microbial product that could survive the rigors of the aerosol environment. Maintaining strain virulence, toxin production, and corresponding lot comparability were critical to successful aerosol delivery.

In 1942, President Roosevelt dedicated an initial 126,720 acres of Utah desert land for use by the War Department. Another biological weapons laboratory was opened 6 days later at Dugway Proving Ground in Utah as a testing and evaluation facility. The remoteness and massive land area of this base was ideal for evaluating how aerosolized biological agents performed in the natural environment. A series of experiments were commenced to evaluate the utility of aerosol dispersal as a means of executing a biological weapon attack, including open-air experiments with active biological agents. Aerosolized organisms were detected as far as 30 or more miles away in large-scale aerosol tests. Clandestine dispersals of surrogate organisms, such as *Serratia marcescens* and *Bacillus globigii* (now *B atrophaeus*) were also conducted in a number of urban locations, including New York and San Francisco. Years later it was realized that these experiments actually resulted in a number of illnesses and possibly at least one death, despite the "harmlessness" of the bacteria used. These tests, while highly unethical, demonstrated the potential for an aerosol attack with a biological weapon.[9–11]

Clinical Exposure Trials: Operation Whitecoat (1954–1973)

Concurrent and subsequent to the massive operational efforts that were underway to produce and evaluate biological agents, limited human clinical studies began with a program called Camp Detrick-22 (CD-22) in 1954. Initially, this program of testing biological agents on human volunteers was to assess incapacitating agents' delivery and effects on soldiers, and was very similar to human evaluation of lachrymatory chemical agents (tear gas). The program was eventually expanded to test the efficacy of medical interventions and vaccines, and became known as Project or Operation Whitecoat. Program volunteers were primarily chosen from US enlisted soldiers who, based on their stated religious preference, were affiliated with the Seventh-day Adventist (SDA) church. These soldiers were promised to serve in the military only in noncombat positions if they were enrolled into Operation Whitecoat as volunteers for testing. In addition to SDA-affiliated soldiers, Ohio State Penitentiary prisoners also attended as volunteers of the program. Both soldiers and the prisoners signed the consent forms before they enrolled to the program. They were free to withdraw from the program any time and they were informed about the possible effects of each study. Overall, more than 2,300 volunteers were tested in 137 protocols to develop and test for safety, vaccines, and therapeutics against tularemia, Q fever, viral encephalitis, Rift Valley fever, sandfly fever, and plague between 1954 and 1973 during Operation Whitecoat.[9] Because this kind of testing is now recognized as unethical, Operation Whitecoat constituted one of the few times in history when aerosolized agent delivery was directly tested in the targeted host, the human being, rather than a surrogate animal species.[12] Although unethically obtained by modern standards, data from these early clinical studies remain highly relevant as true indicators of delivery of biological agents by the aerosol route in humans.

Of the list of potential biological agents tested in this manner, only studies involving Q fever (*Coxiella burnetti*) and tularemia (*Francisella tularensis*) were considered safe enough for use in aerosol challenge in humans. Both agents produced infections that were not rapidly progressive, and antibiotic treatment (ie, chloramphenicol, streptomycin) was readily available

and proven to be effective. Consequently, aerosol studies in humans were performed with these agents, in which a 1-million-liter cloud chamber (Figure 29-1) was employed for the initial aerosol dispersion. This unique structure, with 1-¼-inch-thick steel walls, was truly remarkable in that it was one of the only configuration facilities where small munitions loaded with prepared biological agent could be detonated and aerosol dispersion could be studied over an appreciable amount of time. The black rubber bladders integrated in the otherwise gray exterior of the chamber, which absorbed the percussion from the detonation of the munitions used at the time, gave the enormous sphere its nickname, "Eight Ball." In addition to the study of agent survival estimates, aerosols from the interior could also be used for exposure studies with volunteers from Operation Whitecoat. These controlled clinical exposures were a critical aspect of the ongoing characterization of biological agents because they represented the only opportunity to study the interaction of aerosols originating from detonated munitions with the human respiratory system. These studies provided information on the physical size distribution, biological stability, and corresponding viability of the microbial payloads prepared for delivery on the battlefield. The Eight Ball was used for exposures first with *C burnetii* and then *F tularensis* aerosol trials, and decontaminated in 1970 during the decommissioning of the US offensive biological program. No longer in use, the testing chamber remains at Fort Detrick and was listed in 1977 on the US National Historic Register as a landmark site (National Park Service landmark 77000696). In addition to these aerosol trials, outdoor aerosolized *C burnetti* studies that emulated biological warfare scenarios at Fort Detrick were performed in Dugway Proving Ground, as well.

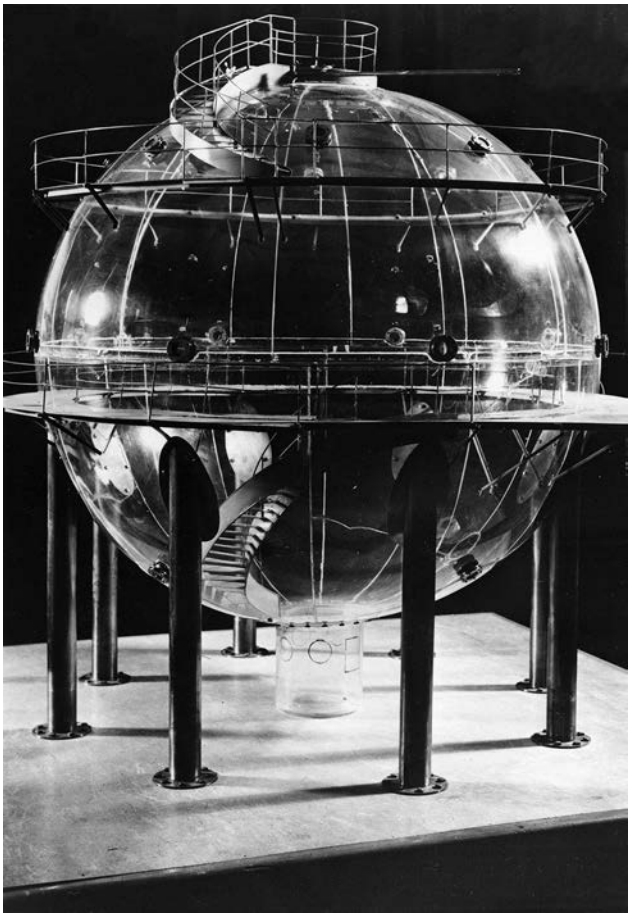


Figure 29-1. Design of the 1-million-liter sphere ball known as "Eight Ball," which was used to expose the Operation Whitecoat volunteers to *Francisella tularensis* and *C burnetii* at Fort Detrick, Maryland.
Photograph reproduced from US Army Medical Department, Medical Research and Materiel Command, Office of Public Affairs, Fort Detrick, MD. R3086, no. 1.

Studies with aerosolized *F tularensis* indicated that when the aerosol residence time increased, infectivity of airborne bacteria decreased. This information, critical to understanding the environmental susceptibility of an organism, opened the door to the development of an attenuated vaccine for *F tularensis*. Early killed and live attenuated tularemia vaccine testing studies with volunteers from the inmates of Ohio State Penitentiary used intracutaneous and respiratory challenge of *F tularensis*. Of the unvaccinated volunteers, 16 of 20 (80%) showed signs of disease following low-dose aerosol challenge ranging from 10 to 52 organisms.[10] Aerosol challenge of vaccinated volunteers resulted in signs of tularemia systemic infection in 8 of 14 (57%) killed vaccine vaccinated volunteers, while only 3 of 18 (16%) live attenuated vaccine vaccinated volunteers had any systemic signs of infection. [10,13]

The potency of an attenuated tularemia vaccine delivered as an aerosol against aerosolized *F tularensis* was tested during follow-up studies.[11] Minor systemic signs and symptoms, such as sore throat and cough, were seen in 30% of aerosolized live vaccine strain (LVS) vaccinated volunteers; pea-sized cervical lymphadenopathy was observed in all vaccinated subjects. Control group and aerosol-vaccinated volunteers were then exposed to approximately 2.5E+04 colony-forming units (CFUs) of aerosolized *F tularensis*; this challenge dose was estimated to be over 2,500-fold more than the minimum dose required to cause disease in humans. Almost all (94%) control group subjects had fever greater than 100°F after a 3- to 5-day incubation period. Following the clinical signs of sudden onset of fever (103°F/104°F), some patients had headache, chills,

and sore throat accompanied by malaise, noticeable myalgia and backache, nausea, and anorexia. Nearly all (89%) of the control group required treatment with antibiotics, while 70% of the vaccinated group had fever and only 23% required treatment. Other delivery routes (oral, cutaneous, and intradermal) for the tularemia vaccine were also evaluated against different challenge routes (intracutaneous, intradermal, aerosol).[13,14]

A similar study was performed to test prophylactic efficacy of tetracycline against aerosolized *F tularensis*.[15] Preceding a 2- to 6-day incubation period, all control group subjects (100%) experienced fever and the other hallmark clinical signs of the disease. The group receiving antibiotic 24 hours postexposure and continuing for 15 days showed no signs of disease. Interestingly, following the cessation of the treatment, 2 of the 10 (20%) volunteers developed acute tularemia. The group that received treatment 28 days initiating 24 hours after exposure did not experience any signs of the disease during or after antibiotic treatment.[10]

A portion of the ongoing clinical efficacy trials with Operation Whitecoat personnel involving Q fever (*C burnetti*) were performed with prisoners from the Maryland State House of Correction.[9] The efficacy of Phase I strain Henzerling and Phase II strain Nine Mile vaccine was tested against aerosolized *C burnetti* in these subjects. These studies indicated that a vaccine of adequate potency was effective in protecting humans against Q fever disease; the protection afforded by these vaccines lasted nearly 1 year after vaccination. Collectively, clinical studies using aerosol infection to develop offensive biological capabilities (at the beginning) and defensive biological capabilities (later) developed and improved medically important countermeasures (vaccines and therapeutic). These studies also contributed to a clear and scientifically realistic understanding of clinical disease progression, signs, symptoms, and diagnostic parameters of many of the priority biological pathogens of interest, namely *C burnetti*, *F tularensis*, sandfly fever, the alphaviral encephalitides, Rift Valley fever, and staphylococcal enterotoxins.[9] This line of investigation also provided clinical insight into the comparative pathophysiology of a disease experimentally induced through a nonnatural route of exposure (aerosol), which was crucial for the viral disease agents that are naturally vector-borne (eg, alphaviruses).

BIOLOGICAL AEROSOL EXPOSURE SYSTEMS

One of the cornerstones in the development of aerobiology capabilities during the former US offensive program and in the present-day defensive biological program is the operational capability to conduct animal studies that incorporate aerosol exposure as a modality for delivering biological agents. In contrast to the clinical studies that took place during the offensive biological program, animal studies presently serve as the only source for data on pathogenesis and performance of medical countermeasures to priority pathogens, such as Tier 1 select agents (those for which there is the most concern regarding their potential for use and the resulting consequences). Appreciation of the componentry in studies involving aerosol challenge is an essential part of the collective required for successful integration into animal experimentation, and remains a core competency of any infectious disease aerobiology program.

Significant efforts to place engineering controls to protect and contain biological aerosols were integrated early and remain the approach in modern facilitates engaged in this type of experimentation. In the modern era in the United States, experimenting with aerosol exposures with select agents requires approval of the Centers for Disease Control and Prevention's (CDC's) Division of Select Agent and Toxins (DSAT) and must follow the recommendations in the *Biosafety in Microbiological and Biomedical Laboratories* (BMBL) manual. Aerosol exposures of animals to infectious agents or toxins, particularly those that are potential biological threat agents, are performed in laboratory environments that are negatively pressurized and rigidly controlled, typically at biosafety level-3 (BSL-3) or higher. Most aerosol exposures are performed inside class III biological safety cabinets (BSCs), which are expensive, completely contained environments with HEPA-filtered supply and exhaust. However, this is not always the case. Some exposures are performed under standard class II BSC or in self-contained equipment, such as the Glas-Col (Terra Haute, IN) inhalation exposure chamber (which is typically used for tuberculosis studies and not select agents and toxins). In some institutions, aerosol exposures are performed in the same room where the exposed animals will be subsequently housed, while in others aerosol exposures take place in separate suites and animals are transported from the holding room to the exposure suite using negatively pressurized transport devices, such as a negatively pressurized and filtered mobile transfer cart. The use of a class III BSC in a separate suite provides the greatest flexibility for decontamination and reuse of the aerosol equipment between multiple pathogens or animal species. The different options also alter the need for personal protective equipment (PPE).

Using aerosols in a class III BSC in a dedicated suite separate from animal holding requires only minimal PPE, while other options, depending on the pathogen, typically require the use of N-95 or powered-air purifying respirators (PAPRs). Beyond the engineering controls and PPE described here, a number of other issues must be considered, including decontamination of the space and security and administrative controls (eg, standard operating procedures, training, and oversight). The laboratory space needed to prepare for aerosol exposures (both the pathogenic agent and the aerosol equipment) and the dose required should be considered and determined.

Exposure Systems

Henderson Apparatus

In 1952, David Henderson described an aerosol exposure system designed for ease of operation that could ensure reproducibility between experiments exposing animals to "clouds" containing infectious organisms.[16] This system also incorporated engineering controls to ensure the safety of those using it, to prevent exposure of laboratory personnel. It consisted of a spraying apparatus (an aerosol generator), an exposure tube (analogous to the exposure chambers used today), and an impinger (an aerosol sampling device), as well as a number of points for monitoring and controlling airflow, vacuum, and pressure. The system was dynamic, with air continuously pumped into and exhausted from the exposure apparatus throughout the exposure to eliminate effects resulting from aerosol decay of the organism, as would occur in a static system. As originally described, the system recirculated waste air that was filtered and reused as dilution air in the exposure.

Modern Exposure Systems

Most aerosol exposure systems used in present-day laboratories that perform bioaerosol studies with select agents are generally some derivation of the original Henderson apparatus.[17] Most are also dynamic and incorporate some measure of safety for laboratory personnel in addition to performing the aerosol in a BSC and with the primary engineering controls described above. These systems are designed with greater flexibility for incorporation of other aerosol generators, exposure chambers, or sampling devices, as well as improved monitoring and control of the aerosol. Exhaust air is filtered but, unlike the Henderson apparatus, waste air is not subsequently recirculated into the exposure loop.

Generators

Although a wide range of aerosol generators can be and are employed, the Collison nebulizer is by far the most commonly used aerosol generator for exposures using select agents (viruses or bacteria) and toxins. This generator has become a standard for three primary reasons: (1) Collison nebulizers are relatively inexpensive and easy to maintain; (2) the Collison generates a relatively uniform, nearly monodisperse particle distribution; and (3) aerosol particles in the size range generated by a Collison (approximately 1 to 2 μm in diameter) will reach the deep lung (alveolar regions) of most mammalian species with minimal deposition in the upper respiratory tract. However, other generators have been used, including in recent years the spinning top aerosol generator (STAG)[18,19] and flow-focusing aerosol generator (FFAG).[20–23] These generators allow for customization to larger particle size distributions, thereby allowing study of differential effects based on deposition in distinct compartments of the respiratory tract. Where it has been examined, in most instances particle deposition in the upper respiratory tract (as compared to deposition in the lower) increases the dose required to cause morbidity and mortality and alters the pathogenesis of the disease, and countermeasures are often more efficacious.[18,21–24] It has been postulated that deposition of encephalitic viruses in the upper respiratory tract might more readily lead to infection of the brain as a result of infection in the olfactory region, but the data accumulated to date is contradictory and needs further examination.[19,25–27]

All aerosol generators described in modern exposure systems utilize "wet," liquid aerosols rather than the dry powder aerosol systems that were used in the past during offensive development. The use of dry powder systems to aerosolize biologically active microbial aerosols raises concerns regarding the potential for "dual-use" research and harkens back to the type of technical expertise common during the now-decommissioned offensive biological development program. Dual-use research is defined by federal policy as, "life sciences research that, based on current understanding, can be reasonably anticipated to provide knowledge, information, products, or technologies that could be directly misapplied to pose a significant threat with broad potential consequences to public health and safety, agricultural crops and other plants, animals, the environment, materiel, or national security."[28] In particular, it is noted among the scope that experiments of concern include those that, "increase the stability, transmissibility, or the ability to disseminate the agent or toxin."[28]

Generation of dry-powder forms of virulent agents has been interpreted as falling into that category; however, that has not precluded the use of dry-powder research in biodefense, and in the pharmaceutical industry there is considerable interest and research into dry-powder delivery of vaccines and therapeutics.[29–37]

Exposure Chamber

The choice of exposure chamber is greatly dictated by the animal species being exposed.[17] Rabbits and nonhuman primates are typically exposed one or two at a time using nose-only or head-only exposure chambers because of the animals' size and laboratory space limitations. Rodents (mice and rats) and ferrets are exposed using either nose-only or whole-body chambers. Nose-only exposure chambers deliver the aerosol to the respiratory tract without contaminating the surface of the animal with the pathogenic organism, alleviating concerns regarding infection via swallowing and/or fomites as opposed to true inhalation. However, the current designs of nose-only systems place far greater stress upon rodents as evidenced by increased corticosterone in the blood that could alter the outcome of infectious disease studies.[38] In addition, recent studies evaluating deposition and retention of select agents and toxins have demonstrated that the majority of what is initially inhaled is removed from the respiratory tract and ends up in the gastrointestinal system.[21–23] The choice of nose-only or whole-body exposure chambers should be carefully considered prior to initiating studies.

Sampling

Traditionally, aerosol sampling of infectious organisms and toxins has been done using liquid impingement as a means to collect a representative sample to quantify both concentration and viability of the pathogenic agent (or activity, if a toxin) in the aerosol at the time of exposure. Impingers come in a variety of shapes and sizes but invariably reply upon impaction of an aerosol into a liquid interface. Impingers allow assessment of viable bacteria or viruses in the aerosol but do not provide a means for assessing particle size or the number of bacteria or viruses per particle. Filters and cyclones are also routinely used for sampling bioaerosols to determine concentration of viable microorganisms.[39–41]

When selecting sampling devices, sampling efficiency should be evaluated, as well as the effects of sampling processes (eg, flow rate, collection media) on the viability of the organism being measured. Particle aerodynamic size can be measured during these types of exposures using either viable impactor-type devices, such as an Andersen cascade collector, or analytical devices that employ dual time-of-flight laser technology. Other optical technologies for particle sizing are also employed, such as laser-scattering type instruments (eg, Grimm Technologies, Incorporated [Douglasville, GA] or TSI Incorporated [Shoreview, MN] particle spectrometers). Size characterization of liquid bioaerosols, whose malleability depends on prevailing environmental conditions in the exposure chamber, and the density of the formulation used are usually expressed as the mass median aerodynamic diameter (MMAD). The MMAD provides a median size of the particle distribution based on the behavior of the particles through the air and the corresponding velocity, rather than an actual physical measurement of size. This type of characterization is appropriate for liquid-based aerosols whose size can be dynamic within the exposure systems. Great care should also be taken to sample in the "breathing zone" where the animal is likely to inhale particles, as particle size could be different outside of that zone. Larger particles can break into smaller particles or shrink through evaporation, which can greatly influence where particles will deposit in the respiratory tract. This can also influence the viability of the microorganism in the aerosol, as has been seen with *F tularensis*, in which increased sodium chloride concentration resulting from particle evaporation resulted in a loss of viability, thereby requiring higher concentrations to achieve a lethal dose.[42] Raising the relative humidity in the chamber improved bacterial viability in the aerosol, but it does not alter infectivity.[42,43] Particle density is also an important consideration because it will influence particle size based on the percentage of solids and volatile components that will be lost due to evaporation.[44]

Monitoring and Control

Older biological aerosol exposure systems allow for monitoring and control of environmental parameters, although typically in a crude fashion via a manually controlled instrumentation panel that must be continuously manipulated by a laboratory aerosol technician operator. Modern biological aerosol exposure systems, in contrast, are operated using fully integrated, process flow-control computer systems in addition to constantly monitoring and recording changes in environmental parameters (relative humidity, temperature, pressure) and flow rates (nebulizer, secondary air, exhaust, sampling).[45] This improves accuracy in exposure timing and control as well as improving dosimetry precision in nonhuman primates. With the increased monitoring

comes the improved ability to control and alter these parameters during the exposure to evaluate the impact on aerosol concentration.

Dosimetry

The most critical aspect of biological aerosol exposure systems is the ability to determine the dose delivered to the exposed animal based on the operational characteristics and performance of the system to the users' requirements. Aerosol "dose" is reported in one of three ways: (1) inhaled (also called presented dose, the total number of infectious organisms or mass of toxin inhaled), (2) deposited (the amount that deposits within the respiratory tract), and (3) retained (the amount that remains in the respiratory tract after a specific time).[17,46] The delineation between deposited and retained dose is time-dependent and is not fixed except within a given system. For example, in 1962 Harper and Morton defined retention of *Bacillus globigii* spores as the number of spores remaining in the lungs of guinea pigs 1 day after an aerosol exposure.[47] A considerable amount of what is inhaled is exhaled, removed, or destroyed by the host's innate mechanisms for clearing the respiratory tract (eg, the mucociliary escalator, mucin, defensins, and surfactants). Deposited and retained dose are difficult to measure for infectious organisms, which begin to replicate or escape from the respiratory tract into the circulation almost as soon as they deposit in the respiratory tract. Further, measuring deposited and retained dose requires sacrificing the animal and harvesting tissues in the respiratory tract. Understanding deposition and retention is useful for understanding the aerosol biology and pathology of infectious organisms, but the impact on the efficacy of medical countermeasures is less clear.[21,22,24,48,49] This must be evaluated on a pathogen- and host-specific basis, and the results from one system should not be generally applied to other systems (including other animal species infected with the same pathogen). Further research is desperately needed.

Parameters Impacting Aerosol Dosimetry

Aerosol Performance

System performance between aerosols is compared using the ratio between the aerosol concentration and the nebulizer concentration, also known as the spray factor. Spray factor can be used to determine the nebulizer concentration required to achieve a desired inhaled dose in future studies with that pathogen. Spray factors can only be compared within a given aerosol system for a particular agent, and different systems are not comparable using spray factor performance. Spray factor is essential to building a microbial database of relative aerosol viability within a particular aerosol system, and ultimately dictates the capability (and limitations) of dosing animal species within an aerosol system with that pathogen. Aerosols performed prior to animal exposures determine the spray factor as well as assess the impact of environmental parameters on aerosol performance. Relative humidity in particular has been shown to impact particle size distribution as well as viability of a number of bacteria and viruses.[43,50–57] Aerosol efficiency (the ratio of viable agent in the aerosol sample to the quantity of agent nebulized) and relative recovery (aerosol sampling of the challenge agent relative to a known standard included in the aerosol) have been used as alternatives to spray factor and serve an equivalent function.[50,58,59] These effects are pathogen- and system-specific and require careful evaluation in developing new systems or working with new pathogens.

Anatomy and Physiology

Numerous studies have highlighted differences in the respiratory anatomy between mammalian species. In particular, the length and degree of branching in the bronchus and bronchioles vary greatly between species, getting smaller in length and with less branching as species get smaller.[60] Differences in the amount of branching have been noted between strains of inbred mice. There is also considerable difference in the thickness of bronchial epithelium and the production of mucus,[61] all factors that can impact particle deposition and retention in the respiratory tract.

Respiratory Function

To determine the inhaled dose requires measuring the respiratory minute volume (Vm) of the experimental animal.[62] For experiments with smaller animals like rodents and ferrets, where multiple animals are exposed at a time, minute volume is determined using a simplistic formula based on the animal's weight and corresponding surface area, and was developed by Arthur Guyton over six decades ago.[63] Other methods to determine respiratory function were developed by Bide and Alexander and are also used.[64,65] Respiratory function is typically measured using plethysmography if larger animals, such as rabbits and nonhuman primates, are employed in experimentation. Plethysmography is typically performed either immediately before or during the aerosol exposure. Plethysmography (and the aerosol exposure) of nonhuman primates is performed while animals are anesthetized, which can dramatically suppress respiratory function. Rabbits,

although not anesthetized, are typically restrained, which can increase respiration and minute volume between rabbits of similar size, age, and gender, and can vary dramatically. Because of space limitations in the class III BSC, plethysmography of rabbits and nonhuman primates can typically be done more easily before the aerosol exposure. If plethysmography is performed prior to exposure, minute volume is presumed not to change during exposure (or changes only minimally), although data have shown tremendous variation in minute volume in larger animals irrespective of weight and other factors such as age, gender, and level of anesthesia. If not accounted for, this can lead to tremendous variation in presented dose delivered to animals during exposure. However, most biological aerosol exposure systems do not account for individual variation in minute volume between animals, instead relying upon a fixed exposure time. This is an area that needs further development to ensure similar and consistent dosing between treated and control groups.

SUMMARY

The interface of aerobiology, infectious disease, and the transmissibility of disease are ever present. Harnessing, controlling, and delivering pathogenic agents by aerosol remains the primary and most predicted route of exposure for both military and state-sponsored terrorist acts using biological weapons. The threat of a biological agent being optimized for aerosol delivery holds the potential to reach a target population more efficiently and more completely than any other possible exposure modality available. Much of what is known in the scientific lexicon of aerobiology, as in many fields of study, is derived primarily from observation of nature and natural processes; namely the transmission of disease either through indirect sources, such as contaminated sewage aerosolized at a particle size distribution that approximates respirability, or direct sources, such as proximal contact with an infected host while coughing or sneezing.[4] The early challenge was to overcome the identified environmental and physiochemical factors that would most rapidly degrade or kill microbial preparations when in an aerosol form. Accordingly, modern development of aerobiological techniques was synthetically modeled after natural processes most efficient at disease transmission. Maintaining the physical characteristics and viability of a pathogenic organism for delivery into the environment by virtue of munitions or secondary direct aerosol generator was no small task, and by all accounts in the history of the offensive biological programs, overcoming these barriers required sophisticated approaches. Early biological weapons programs in the United States and Soviet Union focused initially on transferring laboratory bench-based microbiological propagation into industrial-class operational capability, first producing massive quantities of pathogenic agent. Preparation of live microbiological agents for airborne delivery relied heavily upon techniques for preservation and packaging that maintain viability and protect against environmental degradation once released. Concurrent to developing and perfecting industrial-class microbial propagation, preparation, preservation, and delivery techniques, significant efforts were made to determine pathophysiology and pathogenesis in animal models and even in limited human studies. Complex systems for testing and evaluating optimized microbial preparations using select animal species emerged at this time to better support this effort. Sophisticated testing systems that integrated aerosol delivery to a varied array of animal species developed during this time. The small modular aerosol exposure systems in use in many modern laboratories, which are mere shadows of the industrial versions of the past, continue to function under the same basic design and performance criteria. Conversely, the clinical studies that incorporated aerosol exposure with agents easily treated with available chemotherapeutic agents at the time were an advent that will forever remain in the annals of the offensive biological program. The massive dedication of scientific resources and infrastructure to respond to this effort was specifically focused on aerosol as the primary means of delivery to the enemy. This is an important consideration because a number of the biological agents selected for development were not naturally communicable through the airborne route; therefore no clinical experience with infection existed at the time. Predominant disease models and pathogens that catered to aerosol delivery emerged as cornerstones of the state-sponsored biological weapon programs. These very programs, at their zenith, optimized the industrial production, packaging, and prospective aerosol delivery of biological agents in a manner that history had never witnessed. The aerobiology resources and capabilities adjunctive to the biological weapon programs ultimately experienced a dramatic reduction, and a complete shutdown in many cases, that coincided with the signing of the biological weapons convention in 1969. Some countries, however, continued covert operations, including the aerosol research components, well into the 21st century.

Collectively, in the aftermath of the decommissioning of the offensive biological programs, much of the infrastructure needed to effectively perform research for medical countermeasures was effectively rebuilt, albeit on a much smaller scale with significant technical and engineering limitations in mind. Present-day research organizations that incorporate aerobiology resources and expertise, such as the program at the US Army Medical Research Institute of Infectious Diseases at Fort Detrick, Maryland, embody a small-scale, sophisticated support structure similar to many programs at other federally supported, contracting, and academic laboratories throughout the nation.

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Chapter 30

BIOSAFETY

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SUMMARY

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INTRODUCTION

Biosafety

Biological safety, or "biosafety," is the application of concepts pertaining to risk assessment, engineering technology, personal protective equipment (PPE), policies, training, and preventive medicine to promote safe laboratory practices, procedures, and the proper use of containment equipment and facilities. In biomedicine, laboratory workers apply these tenets to prevent laboratory-acquired infections and the release of pathogenic organisms into the environment. A biohazard is defined as any microorganism (including, but not limited to, bacteria, viruses, fungi, rickettsiae, or protozoa); parasite; vector; biological toxin; infectious substance; or any naturally occurring, bioengineered, or synthesized component of any such microorganism or infectious substance that is capable of causing the following:

- death, disease, or other biological malfunction in humans, animals, plants, or other living organisms;
- deleterious alteration of the environment; or
- an adverse impact on commerce or trade agreements.

These hazardous agents may be handled safely through careful integration of accepted microbiological practices and primary and secondary containments of potential biohazards.

Primary containment involves placing a barrier at the level of the hazard, confining the material to protect laboratory personnel and the immediate laboratory environment by adhering to prudent laboratory practices and appropriate use of engineering controls. Examples of primary containment include biological safety cabinets (BSCs), ventilated animal cages, and associated equipment. Secondary containment involves protecting the environment external to the laboratory from exposure to infectious or biohazardous materials through facility design and operational practices.

Combinations of laboratory practices, containment equipment, and special laboratory design are used to achieve different levels of physical containment. The current terminology in the United States is "biosafety level" (BSL), though historically, the designation "P" was used to indicate the level of physical containment (such as P-1 through P-4).[1] BSL is used in *Biosafety in Microbiological and Biomedical Laboratories*,[1] which focuses on protecting laboratory employees. Biosafety level may also be abbreviated "BL," which is used in Appendix G of the National Institutes of Health (NIH) publication Guidelines for Research Involving Recombinant DNA Molecules (also known as the NIH Guidelines).[2] However, Appendix G of the NIH Guidelines focuses primarily on physical containment involving work with recombinant deoxyribonucleic acid (DNA) molecules and organisms and viruses containing recombinant DNA molecules.

There are four levels of biosafety (designated 1 through 4) that define the parameters of containment necessary to protect personnel and the environment.[1] BSL-1 is the least restrictive, whereas BSL-4 requires a special containment or maximum containment laboratory facility. Positive-pressure encapsulating suits (PPES), primarily manufactured by ILC Dover (ILC Dover LP, Frederica, DE) or Honeywell Safety Products (Smithfield, RI), or gas-tight Class III BSC systems are used in a maximum containment (BSL-4) laboratory environment. Biosafety is not possible without proper and extensive training. The principal investigator or laboratory supervisor is responsible for providing or arranging for appropriate training of all personnel within the laboratory to maintain and sustain a safe working environment.

Evolution of Biosafety

Steps to limit the spread of infection have been practiced in the field of biomedicine since human illness was associated with infectious microorganisms and biologically derived toxins. However, Fort Detrick (in Frederick, MD) is considered the birthplace (beginning in the 1940s) of modern biosafety as a discrete discipline. During the early years of biosafety, safer working practices, principles, and engineering controls were developed,[3,4] as individuals conducting biomedical research commonly became infected with the organism being studied. As the hazard of working with organisms increased, so did the need to protect laboratory personnel conducting the research. Contributions to the field of biosafety were a direct result of the innovations and extensive experiences of Fort Detrick personnel who worked with a variety of infectious microorganisms and biological toxins. Dr Arnold Wedum, director of industrial health and safety at Fort Detrick—and regarded by many as the father of the US biosafety profession—promoted the attitude that biosafety should be an integral part of biomedical research.[5]

To enhance worker safety and environmental protection, Wedum[4] promoted use of the following:

- class III gas-tight BSC;
- noninfectious microorganisms in recombinant DNA research;
- P-4 (today's BSL-4) principles, practices, and PPES facilities when working with potentially aerosol-transmitted zoonotic microorganisms (eg, those causing tularemia and Q fever if a class III cabinet system was not available); and
- vaccination or immunization of laboratory workers.

Another safety enhancement was demonstrating and publicizing the importance of prohibiting mouth pipetting for fluid transfers involving hazardous material.[6,7] Dr Emmett Barkley[8] reiterated the hazard of oral pipetting, which should not be practiced in the laboratory. Barkley was chief of the Safety Division of the National Cancer Institute (Bethesda, MD) and subsequently director of research safety at NIH when the NIH Guidelines were developed and adopted. He was instrumental in developing physical containment parameters for recombinant DNA research.[9]

Critical to the advancement of modern biosafety was the development of air filtration technology. During the early 1940s, the US Army Chemical Warfare Service Laboratories (Edgewood, MD) studied the composition of filter paper captured from German gas mask canisters in search of better smoke filters. These early studies resulted in the design of collective protection filter units for use at the particulate-removal stage by a combined chemical, biological, and radiological purification unit of the US armed services. In the late 1940s, the Atomic Energy Commission (precursor of the Nuclear Regulatory Commission) adopted this type of filter to confine airborne radioactive particles in the exhaust ventilation systems of experimental reactors and in other areas of nuclear research. Subsequently, Arthur D Little Company, Incorporated (Boston, MA) and the US Naval Research Laboratory (Washington, DC) developed a prototype glass-fiber filter paper. Eventually, thin, corrugated, aluminum-alloy separators replaced the original asbestos, thermoplastics, and resin-treated papers. Throughout this development period, military specifications were developed and implemented to ensure the safe operating and optimal conditions of filters,[10] ultimately leading to the production of high-efficiency particulate air (HEPA) filters, which are used today in a variety of engineering controls, as well as in laboratory heating, ventilation, and air conditioning systems.

HEPA filters are constructed of paper-thin sheets of borosilicate medium that are pleated to increase their surface area. The borosilicate sheets are tightly pleated over aluminum separators for added stability and affixed to a frame.[10] A BSC first developed in 1964 for a pharmaceutical company used HEPA filter technology to provide clean air in the work area and containment as the primary barrier placed at the source of hazardous powders. Subsequent research led to the development of a class II, type A BSC that was delivered to the National Cancer Institute by the Baker Company (Sanford, ME).[11] The National Cancer Institute also developed a specification for the first class II, type B console BSC. HEPA filters have been proven to be effective, economical, and reliable devices for removing radioactive and nonradioactive particulate aerosols at a high rate of collection frequency.[10]

Operation and retention efficiency of HEPA filters has been documented. Three mechanisms account for the collection (retention) of particles within HEPA filters:

1. Small particles ranging from 0.01 to 0.2 μm in diameter are collected in a HEPA filter by diffusion and are retained at an efficiency approaching 100%.
2. Particles in the respirable range (those of a size that may be inhaled and retained in the lungs, 0.5 to 5.0 μm in diameter) are retained in a HEPA filter by a combination of impaction and interception at an efficiency approaching 100%.
3. Particles with an intermediate size range (between 0.2 and 0.5 μm in diameter) are retained by a combination of diffusion and impaction.

The HEPA filter is least efficient at retaining particles with a diameter of 0.3 μm, with a minimum collection efficiency of 99.97%. Hence, a standard test of HEPA filter efficiency uses a generated aerosol of particles that are 0.3 μm in diameter; to pass the test, the HEPA filter must retain 99.97% of the particles.[12]

All the air exhausted from BSCs, within which infectious materials must be manipulated, is directed through a HEPA filter before recirculation to a laboratory room or discharge to the outside environment through the building exhaust system. Therefore, in addition to adherence to rigorous work practice controls, HEPA filtration of laboratory exhaust air provides an extra margin of safety for workers, the laboratory areas, and the outside environment.

RISK GROUPS AND BIOSAFETY LEVELS

Risk Groups

Agents infectious to humans, including those used in research, are placed into risk groups based on the danger they pose to human health. Risk group assignment helps researchers determine the containment condition (or BSL) appropriate for handling a particular agent (Table 30-1). Multiple schemes for assigning risk groups have been developed. The NIH Guidelines, Health Canada (Ottawa, Ontario, Canada),[13] other nations, and the World Health Organization (Geneva, Switzerland)[14] all have risk group paradigms. The World Health Organization has categorized infectious agents and biological toxins into four risk groups. These risk groups relate to, but do not equate to, the BSLs of laboratories designed to work with organisms in each risk group.[14] Risk group 1 (no or low individual and community risk) comprises microorganisms including *Escherichia coli* K12 and *Candida albicans* that are unlikely to cause human or animal disease in healthy adult individuals. Risk group 2 (moderate individual risk, low community risk) includes pathogens that can cause human or animal disease, but are unlikely to be serious hazards to laboratory workers, the community, livestock, or the environment. Laboratory exposures may cause serious infection, but effective treatment and preventive measures are available, and the risk of infection spreading is limited. An example is a causative agent of viral hepatitis, Hepatitis B virus, in humans and animals. Risk group 3 (high individual risk, low community risk) includes pathogens that usually cause serious human or animal disease, but do not ordinarily spread from one infected individual to another efficiently. Effective treatment and preventive measures are likely available. An example is the causative agent of tularemia, *Francisella tularensis*, in humans and animals. Risk group 4 (high individual and community risk) pathogens usually cause serious human or animal disease and can be readily transmitted from one individual to another, either directly or indirectly. Effective treatment and preventive measures are not normally available. Examples include Variola virus, Ebola virus, Lassa fever virus, and Marburg fever virus.

Assigning Agents to Risk Groups

It is important to understand how microorganisms are placed in risk groups and how that knowledge is used to develop procedures and physical infrastructure to contain these agents. The following criteria must be considered to assess risk while working in a laboratory or animal environment with a specific microorganism.

- **Number of past laboratory infections**. The most frequent laboratory-associated infections in humans are caused by the Brucella species. Extra caution must be taken when working with this agent because of its low

TABLE 30-1

RELATIONSHIPS OF RISK GROUPS, BIOSAFETY LEVELS, PRACTICES, AND EQUIPMENT

Risk Group	Biosafety Level	Laboratory Type	Laboratory Practices	Safety Equipment
1	Basic: BSL-1	Basic teaching; research	Good microbiological techniques	None; open bench work
2	Basic: BSL-2	Primary health services; diagnostic services; research	Universal precautions plus protective clothing and biohazard sign	Open bench plus BSC for potential aerosols
3	Containment: BSL-3	Special diagnostic services; research	As level 2 plus controlled access, double door entry, special clothing, and directional airflow	BSC and/or other primary devices for all activities
4	Maximum containment: BSL-4	Dangerous pathogens; research	As level 3 plus airlock entry, shower exit and special waste disposal	Class III BSC, or positive-pressure protective suits in conjunction with class II BSCs, double-door autoclave (through the wall), and filtered air

BSC: biological safety cabinet
BSL: biosafety level

TABLE 30-2

CASE-FATALITY RATE BY DISEASE

Disease (Untreated)	Organism	Case-Fatality Rate
Plague, bubonic	*Yersinia pestis*	50%–60%
Cholera	*Vibrio cholerae*	50% or more
Tularemia, pulmonary	*Francisella tularensis*	30%–60%
Anthrax, cutaneous	*Bacillus anthracis*	5%–20%
Tularemia, typhoidal	*Francisella tularensis*	5%–15%
Brucellosis	*Brucella* species	2% or less
Q fever	*Coxiella burnetii*	1%–2.4%

infectious dose for humans. About 10 to 100 organisms can cause an infection in a susceptible human host.[15]

- **Natural mortality rate**. The natural mortality or case-fatality rate of diseases varies widely (Table 30-2).[15]
- **Human infectious dose**. Working with an organism having a low infectious dose for humans will place the laboratory worker at a greater risk than working with an organism having a higher infectious dose. The infectious dose of organisms for humans varies and is also dependent on the immunological competency of the host (Table 30-3). Although the literature contains information about the potential infectious dose for humans as extrapolated from animal data (see Table 30-3), an attempt to provide quantitative human infectious doses is not possible.[16]
- **Efficacy of vaccination and treatment (if available)**. Vaccines are available for some of the agents studied within the laboratory. Receiving a vaccination must be based on a risk assessment. Only those individuals who are considered at risk should be offered the vaccination. However, the potential risk of the adverse effects from the vaccination might outweigh the risk of acquiring an infection. In addition, a vaccination might not provide 100% protection; an overwhelming infectious dose can overcome the protective capacity of a vaccination. Therefore, a vaccination should be considered only as an adjunct to safety, not as a substitute for safety and prudent practices. Treatment (chemoprophylaxis) in the form of antibiotic therapy may also be available to treat illnesses caused by many of the microorganisms being manipulated in the laboratory, specifically by the bacterial and rickettsial agents. It is necessary to determine the antibiotic sensitivity and resistance pattern (antibiogram) of the agent under investigation. The rationale is that treatment will be known in advance if an inadvertent laboratory exposure occurs. Treatment for exposure to a virus might be problematic because only symptomatic treatment may be available. There are few available antiviral agents that may be effective for postexposure prophylaxis. Specific antiviral agents include the following:
  - Rabies: rabies immune globulin for passive therapy, followed by the human diploid cell rabies vaccine or rabies vaccine, adsorbed for active vaccination.
  - Macacine herpesvirus 1 (formerly Cercopithecine herpesvirus; B virus): valacyclovir hydrochloride (VALTREX; GlaxoSmithKline, Research Triangle Park, NC).
  - Arenaviridae and bunyaviridae (including the viruses that cause Lassa fever, Argentine hemorrhagic fever, and Crimean-Congo hemorrhagic fever): ribavirin. This material can be used under an investigational new drug protocol (in the United States)

TABLE 30-3

HUMAN INFECTIOUS DOSE BY ORGANISM

Organism	Infectious Dose	Route of Exposure
Vibrio cholerae	108	Ingestion[1]
Yersinia pestis	100–20,000	Inhalation[2]
Bacillus anthracis	~ 1,300	Inhalation[3]
Brucella species	10–500	Inhalation[2]
Francisella tularensis	10	Inhalation[4]
Coxiella burnetii	1	Inhalation[5]

Data sources: (1) Sack DA, Sack RB, Nair GB, Siddique AK. Cholera. *Lancet*. 2004;363:223–233. (2) Franz DR, Jahrling PB, Friedlander AM, et al. Clinical recognition and management of patients exposed to biological warfare agents. *JAMA*. 1997;278:399–411. (3) Dull PM, Wilson KE, Kournikakis B, et al. *Bacillus anthracis* aerosolization associated with a contaminated mail sorting machine. *Emerg Infect Dis*. 2002;8:1044–1047. (4) Jones RM, Nicas M, Hubbard A, Sylvester MD, Reingold A. The infectious dose of *Francisella tularensis* (tularemia). *Appl Biosafety*. 2005;10:227–239. (5) Jones RM, Nicas N, Hubbard A, Reingold A. The infectious dose of *Coxiella burnetti* (Q-fever). *Appl Biosafety*. 2006;11:32–41.

only for empirical treatment of hemorrhagic fever virus patients while awaiting identification of the etiological agent.
    - Poxviridae (including the viruses that cause smallpox and monkeypox): in addition to the Dryvax (Wyeth Inc, Philadelphia, PA) vaccine variant derived from Vero cells, ACAM2000 (Acambis, Canton, MA), two small molecule poxvirus inhibitors are currently in clinical trials: the Cidofovir lipid conjugate CMX001 (Chimerix Pharmaceuticals, Durham, NC) and ST-246 (Tecovirimat; SIGA Technologies, New York, NY).[17–19]
    - Retroviridae (including human immunodeficiency virus): the latest highly active antiretroviral therapy recommendations for postexposure prophylaxis are available through the US Public Health Service.[20]
    - There are currently small-molecule therapeutics under development for treating potential filoviradae (including Marburg and Ebola viruses) infection, but no products have yet been approved by the US Food and Drug Administration for clinical use.[21]
    - Additional vaccines and antiviral treatments for flaviviradae and togaviradae infections are in varying stages of development or clinical trials. A US Food and Drug Administration licensed vaccine is available as a preventative treatment or prophylaxis against such agents as Japanese encephalitis virus and Yellow Fever virus, while other vaccines remain under investigational new drug status for Venezuelan equine encephalitis, Dengue fever, and tick-borne encephalitis virus.[22–25]
- **Extent to which infected animals transmit the disease**. This discussion involves the zoonotic diseases or diseases that can be transmitted from animals to humans. These diseases include, but are not limited to, the following:
    - those transmitted directly from animals to humans (eg, rabies);
    - those that can be acquired indirectly by humans through ingestion, inhalation, or contact with infected animal products, soil, water, or other environmental surfaces that have been contaminated with animal waste or a dead animal (eg, anthrax); and
    - a disease that has an animal reservoir, but requires a mosquito or other arthropod to transmit the disease to humans (eg, St Louis encephalitis virus and Rocky Mountain spotted fever).

    Exposure risks in laboratories that use animals may differ from the exposure risks encountered in microbiology laboratories. Within microbiology laboratories, hazardous conditions may arise from human activities or from equipment within the laboratory. In animal facilities, the animals themselves may create hazards for the laboratory workers via:
    - generation of infectious aerosols;
    - animal bites or scratches to the person handling the animal; and
    - shedding of infectious known or unknown zoonotic agents in animal secretions and excretions, contaminating the animal holding room, cage, bedding, equipment, or other fomites. For example, in addition to usual activities in the laboratory, handling materials contaminated with hantaviruses is a concern because viruses are spread as aerosols or dusts from rodent urine, droppings, or by direct contact with saliva through cuts or mucous membranes.
- **Stability of the agent**. An agent's (microorganism's) stability to environmental conditions and susceptibility or resistance to disinfectants results from its internal and external chemical composition. For instance, spores of the genus *Bacillus* are resistant to adverse environmental conditions and disinfectants in part because of the presence of dipicolinic acid (pyridine-2,6-dicarboxylic acid) in their spore coat. Dipicolinic acid plays a significant role in the survival of *Bacillus* spores exposed to wet heat and ultraviolet radiation.[26] Many viruses and bacteria are sensitive to environmental conditions and disinfectants because of the high lipid content in their outermost layer.

Biosafety Levels

BSLs are guidelines that have evolved to protect laboratory workers from biological hazards. They do not take into account additional hazards found within the laboratory, including chemical, physical, or radiological hazards. These guidelines are based on data from laboratory-acquired infections and on an understanding of the risks associated with various manipulations of many agents transmissible by different routes. These guidelines operate on the premise that safe work sites result from a combination of engineering controls, management policies, work practices and procedures, and, occasionally, medical interventions. The different BSLs developed for microbiological and biomedical

laboratories provide increasing levels of personnel and environmental protection.[1] BSL descriptions comprise a combination of facilities, equipment, and procedures used to handle infectious agents to protect the laboratory worker, the environment, and the community. This combination is proportional to the potential hazard level (risk group) of a given infectious agent. Equipment serving as primary barriers includes but is not limited to BSCs, centrifuge safety cups, and containment animal caging. Facilities also consist of secondary barriers, such as self-closing/locking doors, hand-washing sinks, and unidirectional airflow from the least hazardous areas to the potentially most hazardous areas. Procedures consist of standard and special microbiological practices. Finally, PPE includes dedicated laboratory clothing and respiratory protection.

There are four BSLs described in *Biosafety in Microbiological and Biomedical Laboratories*.[1] These levels range from a basic level (BSL-1) through maximum containment (BSL-4). BSL-1 consists of facilities, equipment, and procedures suitable for work, with infectious agents of no known or of minimal potential hazard to healthy laboratory personnel. BSL-1 represents a basic level of containment that relies on standard microbiological practices, with no special primary or secondary barriers recommended, other than a sink for hand washing.

BSL-2 consists of facilities, equipment, and procedures applicable to clinical, diagnostic, or teaching laboratories; suitable for work involving indigenous moderate-risk infectious agents present in the community; and associated with human disease of varying severity for which vaccines or therapeutics are usually available.[1]

Primary hazards to personnel working with these agents are accidental percutaneous or mucous membrane exposures and ingestion of infectious materials. (Inhalation exposure to agents at the BSL-2 level is uncommon; the main risk with aerosol generation is potential contamination of the laboratory with infectious agents that can result in exposure through breaks in the skin, ingestion, or injury. Therefore, all aerosol-generating procedures should be performed in a BSC or other primary containment equipment, but respiratory protection to mitigate aerosol exposure is rarely recommended unless there are other circumstances involved.) BSL-2 differs from BSL-1 in five ways:

1. Laboratory personnel receive specific training in handling pathogenic agents.
2. Scientists experienced in handling specific agents direct the laboratory.
3. Access to the laboratory is limited when work is in progress.
4. A laboratory-specific biosafety manual is prepared or adopted.
5. Procedures capable of generating potentially infectious aerosols are conducted within class I or class II BSCs or other primary containment equipment. Personnel receive specific training in the proper use of primary containment equipment and adhere strictly to recommended microbiological practices.

BSL-3 includes facilities, equipment, and procedures applicable to clinical, diagnostic, research, or production facilities in which work is done with indigenous or exotic agents that may cause serious or potentially lethal disease, especially after inhalation exposure, and vaccines or therapeutics may be available.[1] Hazards to personnel working with these agents include autoinoculation, ingestion, and exposure to infectious aerosols. BSL-3 differs from BSL-2 in four ways:

1. At BSL-3, laboratory personnel receive more extensive training in handling potentially lethal pathogenic agents than the degree of training received at BSL-2.
2. All manipulations of infectious or toxin-containing materials are conducted within class II or class III BSCs or other primary containment equipment. Personnel are trained to use this safety equipment properly.
3. The laboratory has special engineering and design features that include access zones with two self-closing and locking doors, sealed penetrations or penetrations capable of being sealed, and directional airflow (from areas of low-hazard potential to areas of high-hazard potential). Laboratory personnel are trained to understand these special design features.
4. Only the laboratory director can approve a modification of these BSL-3 recommendations.

BSL-4 comprises facilities, equipment, and procedures required for work with dangerous and exotic agents that pose a high individual risk of life-threatening disease transmitted by the inhalation route and for which a vaccine or therapy are not usually available.[1] Hazards to personnel working with these agents include autoinoculation, mucous membrane or broken skin exposure to infectious droplets, and exposure to infectious aerosols. BSL-4 differs from BSL-3 in six ways:

1. Laboratory personnel receive specific and thorough training to handle extremely hazardous infectious agents. Their supervisors are competent scientists who are trained and experienced in working with these agents.
2. Laboratory personnel understand the function of primary and secondary barriers and laboratory design features. They are trained in standard and special microbiological practices and the proper use of primary containment equipment.
3. The laboratory director strictly controls access to the laboratory.
4. The laboratory is in a controlled area within a building, completely isolated from all other areas of the building, or is in a separate building.
5. All activities involving agent manipulation within the work areas of the laboratory are conducted within a class III BSC, or within a class I or class II BSC used in conjunction with a one-piece, positive-pressure protective suit that is ventilated by a life-support system.
6. The BSL-4 laboratory, or maximum containment laboratory, has special engineering and design features to prevent dissemination of microorganisms to the environment.

BIOSAFETY PROGRAM ELEMENTS REQUIRED FOR CONTAINMENT AND MAXIMUM CONTAINMENT LABORATORIES

Measures Taken in Research to Protect Laboratory Workers

Although BSL-3 practices, safety equipment, and facility design and construction are applicable to clinical, diagnostic, teaching, research, and production (large-scale) facilities, where work is done with indigenous or exotic agents with the potential for respiratory transmission and lethal infection, this section will emphasize BSL-3 research laboratories. BSL-4 practices, safety equipment, and facility design and construction apply to work in a reference diagnostic or research setting with dangerous and exotic agents that pose a high individual risk of life threatening disease. These agents may be transmitted by aerosol, and there may be no available vaccine or therapy. BSL-4 research facilities, both class III BSC laboratories and protective-suit laboratories, will be covered in this section. Due to the Biological and Toxin Weapons Convention of 1972, legitimate production (large-scale or greater than 10 L of culture) BSL-4 facilities do not currently exist (most BSL-4 operations are small scale only because of the working conditions inherent to a BSL-4 suit or cabinet laboratory; large-scale facilities would be used only in very special circumstances and do not exist in the United States).

Documenting Safety Procedures

A laboratory's biological safety program manual is a laboratory-specific guide that should include safety standards and standing operating procedures (SOPs), guidelines, and documents for the containment laboratory (see the Lawrence Berkeley National Laboratory's *Biological Safety Program Manual*[27] as an example). These safety SOPs identify the special hazards of the laboratory and the procedures to abate or mitigate the associated risk. SOPs or documents specify the following:

- laboratory entry and exit in detail;
- proper use of laboratory-specific safety equipment (eg, BSCs, sterilizers, pass boxes, and dunk tanks);
- decontamination procedures for the specific laboratory;
- maintenance of laboratory safety and maintenance-related records (access logs, drain flush logs, emergency deluge shower, and eyewash periodic test logs);
- floor plan with hand-wash sinks and all other safety features annotated;
- emergency and routine communication procedures for the specific laboratory; and
- laboratory and agent-specific training for all laboratory personnel.

A compilation of existing SOPs, specifying how a laboratory worker would access the SOPs (online, paper copy in a binder, or both) is suggested. To meet the specific training and proficiency requirement, trainers should provide documentation for standard safety and laboratory essential training, with specific additions for the laboratory that cover orientation for workers new to the laboratory and laboratory-unique procedures and operations. Trainers should consider including in the manual material safety data sheets for the chemicals used in the laboratory. Material safety data sheets for chemicals can be obtained from vendors' websites or from the institutional chemical hygiene officer.

Assessing Individual Risk

For each person working in a BSL-3 and BSL-4 research laboratory, a supervisor conducts a detailed, thorough, individually tailored job hazard analysis or workplace hazard analysis (risk assessment). During

this analysis, each task the individual intends to perform within containment is evaluated in terms of its inherent risk (as described in Risk Groups and Biosafety Levels, above). Each task is considered in terms of a potential laboratory exposure to the infectious agent (and its associated toxins for toxin-producing [toxigenic] agents). Considerations include use of sharp instruments and animals that could potentially result in puncture injuries, operations that may generate infectious aerosols, and direct handling of infectious agents versus observing (auditing) others working with biological materials. The hazards, once identified, are mitigated, preferably by isolating operations that pose a risk within primary and secondary containment devices (barriers), by substituting unbreakable plastic laboratory vessels for glassware and blunt instruments for sharp instruments, and by chemically or physically immobilizing animals to prevent or reduce the risk of sudden or unpredictable behavior leading to bites and scratches. Once the risk assessment is written, this document is approved by the second-line supervisor and reviewed by both the biological safety officer and the occupational health physician for accuracy and completeness.

The preferred means to mitigate risk is by using engineering controls (eg, BSCs, chemical fume hoods, sealed centrifuge rotors, and safety cups) and partial containment caging for animals (eg, micro isolator cages; ventilated cage racks; and ventilated, negative-pressure, HEPA-filtered rigid cubicles or flexible isolators). Where the hazard cannot be eliminated by physical means, it can be managed by administrative controls that provide specific training on procedures. Examples of such procedures include disposing used injection needles without recapping them or using an approved, one-handed practice to recap needles, either the one-handed scoop technique or a one-handed technique using a recapping device (an engineering control that holds the cap in place). Specific training is provided to encourage workers to use safe methods and operations to prevent aerosol generation, skin and mucosal contact with infectious agents, and handling of sharps where they cannot be eliminated.

If the hazard cannot be eliminated by engineering or administrative controls, it may be mitigated by using PPE to protect against contact, as well as mucosal and respiratory exposure. Vaccinations, when available and where medically indicated, may serve as an adjunct to PPE, but never as a substitution for PPE. Once all the tasks an individual will perform have been assessed and all the infectious and toxic agents the individual will work with have been identified, the tasks and agents are recorded in a document that the worker and the supervisor prepare together. The mitigating controls are then chosen with input from safety professionals and occupational health and medical staff to form a collection of primary barriers, approved practices, PPE, and vaccinations. Based on an individual worker's current educational and experience levels and state of health, certain controls may not be feasible. High-risk tasks may have to be avoided, on a spectrum that may range from observing high-risk tasks (in-vivo work, such as manipulations of exposed animals) and performing low-risk tasks (in-vitro work with infected cell cultures in a BSC), to the extreme that the individual may not be granted access to the containment laboratory.

Physical Barriers

Primary barriers include class II and class III BSCs, PPESs, and containment animal housing. Class II BSCs are open-fronted cabinets with HEPA filtered laminar airflow. Class II type A1 and type A2 cabinets may exhaust HEPA-filtered air back into the laboratory or may exhaust the air to the environment through an exhaust canopy. Class II type B1 cabinets have HEPA-filtered down-flow air composed of uncontaminated, recirculated in-flow air (30%) and exhaust most (70%) of the contaminated air through a dedicated duct with a HEPA filter to the atmosphere. Class II type B2 (total exhaust) cabinets exhaust all in-flow and down-flow air to the atmosphere after passing through a HEPA filter located in a dedicated exhaust duct. To verify proper operation, all class II BSCs must be field certified in accordance with National Sanitation Foundation International Standard/American National Standard for Biosafety Cabinetry Class II (Laminar Flow) Biosafety Cabinetry Standard 49[28] on initial installation, at least annually thereafter, or after every major repair or relocation of the cabinet.[1,29,30] It is recommended that accredited certifiers be engaged for provision of class II BSC certification and repair service. Class II cabinets may be used in BSL-3 laboratories, when supplemented by use of PPE (gloves, gowns, and respiratory protection when warranted by a risk assessment), and may be used in BSL-4 laboratories in conjunction with wearing a one-piece, positive-pressure, ventilated suit with a life-support system, an in-line HEPA or high-purity filter, and supplied with grade D breathing air.[31]

When working within a class II BSC, the equipment and materials are arranged in a clean-to-dirty layout, with clean materials in the center of the workspace, potentially contaminated materials at one end of the workspace within the cabinet, and potentially contaminated waste materials at the other end of the workspace.[32] Class III cabinets are totally enclosed, ventilated, gas-tight cabinets. They provide the highest level of product, personal, and environmental

protection against respiratory exposure to infectious or toxic aerosols and are most suitable for work in BSL-3 and BSL-4 laboratories. Operations are conducted using shoulder-length gloves or half-suits connected to the cabinets. Air is supplied to the class III cabinet through a HEPA filter, and air exhausted from the cabinet to the atmosphere passes through two HEPA filters in series (or one HEPA filter and an exhaust air incinerator). Materials are removed from the cabinet by passing them through an interlocked, double-door sterilizer or through a chemical dunk tank filled with an appropriate disinfectant for the infectious agents or toxins in use at BSL-4, but some class III cabinets interlock with a class II BSC for removal at BSL-3. Several class III cabinets, housing a refrigerator, cell culture incubator, centrifuge, or aerosol-generating equipment, may be connected in a cabinet line as an integrated system for use in a BSL-3 laboratory or in a BSL-4 cabinet laboratory. A complete change of clothing is required for workers, including a dedicated laboratory scrub suit, jumpsuit or gown, shoes, and examination gloves for hand protection in case of a puncture or if a pinhole develops in the cabinet shoulder-length gloves, or half-suits.

Primary barriers for animal housing include the following: (*a*) micro isolator cages with filter tops for rodents; (*b*) ventilated rodent cage racks; (*c*) ventilated, negative-pressure, HEPA-filtered cubicles; (*d*) ventilated, negative-pressure, HEPA-filtered flexible film isolators; and (*e*) rigid, ventilated, negative-pressure, HEPA-filtered isolation cages.[32] Rigid, ventilated, negative-pressure, HEPA-filtered, mobile animal transport carts have been developed at US Army Medical Research Institute of Infectious Diseases to isolate animals during transfer between containment animal facilities.[33] Other primary containment devices include ventilated, filtered enclosures for continuous flow centrifuges and use of sealed rotors and centrifuge safety cups in conventional centrifuges. Primary containment devices used in necropsy rooms include downdraft necropsy tables, specially designed class II cabinets for conducting necropsies, and HEPA-filtered vacuum shrouds for oscillating bone saws.

Personal Protective Equipment

In BSL-3 containment, laboratory workers wear protective clothing, such as solid-front or wraparound gowns, scrub suits, or coveralls. This protective clothing is not to be worn outside the laboratory. To aid in enforcement of this rule, laboratory clothing may be color-coded, so that it can be readily identified if worn outside the laboratory. Scrub suits are typically two-piece ensembles composed of trousers and tunics. Tunics with long sleeves that terminate in knit wrist cuffs aid in donning protective gloves. Gloves are drawn over the cuffs and may be secured in place using tape. Long-sleeved tunics are favored over short-sleeved tunics because long sleeves with gloves taped to the sleeves can provide a physical barrier to protect the skin of the wrists and arms from potential exposure to infectious agents, including bacterial spores.[33] Disposable clothing should not be reused. Reusable clothing is decontaminated, usually by autoclaving, before being laundered to prevent an exposure hazard to laundry workers.[30,34] Clothing is changed when overtly contaminated or after every work session, depending on facility policy. The wearing of dedicated laboratory shoes or safety shoes may be required in BSL-3 facilities. Otherwise, disposable shoe covers should be worn. Wearing dedicated laboratory socks provides comfort to the feet and extra skin protection to exposed ankles if trousers are not long enough to cover the legs fully. Not all biocontainment facilities in the United States require workers to have a change of clothes. If a clothing change is required, dedicated socks and shoes are indicated. In the absence of a clothing change requirement, the dedicated shoes and socks may not be used in lieu of shoe covers, coveralls, or no additional PPE, depending on a risk assessment.

Protective gloves must be worn when handling infectious materials, animals, and contaminated material. Gloves are selected to meet the needs of the risk assessment. Nitrile or latex gloves may be appropriate if they provide the worker with protection from the infectious agent being handled. However, gloves manufactured from other materials (eg, neoprene [DuPont Performance Elastomers LLC, Wilmington, DE], butyl rubber, and Hypalon [DuPont Performance Elastomers LLC]) may be indicated to protect against exposure to other contaminated materials, such as toxins, organic solvents, and caustics, or to serve as an alternative to personnel who may have allergic reactions or sensitivities to latex or nitrile. Gloves should be changed frequently, followed by thorough hand washing. Disposable gloves should never be reused. To ensure protection when working with highly hazardous materials, double gloving (wearing two pairs of gloves) should be practiced, with the inner glove taped to the wrist cuff to minimize potential contamination. If the outer glove is punctured or torn, the protective skin barrier should still be maintained by the inner glove if it was not breached (provision of redundant protection). If working with contaminated sharps (eg, needles, scalpels, glass slides, capillary tubes, pipettes) or with infected animals that may bite or scratch, laboratory workers should consider wearing cut-resistant over-gloves (eg, Kevlar [EI Du Pont de Nemours and

Company, Wilmington, DE]; armored, stainless-steel mesh; or leather gloves) for additional protection.[35] If working with materials where there is a splash hazard, the use of safety goggles or face shields and head covers (bonnets, caps, hood) may be indicated, unless the individuals are using a full-face respirator, such as a powered air-purifying respirator (PAPR).

When entering rooms housing infected animals, additional PPE (wraparound gowns or Tyvek [DuPont Tyvek, Richmond, VA] coveralls, foot covers or boots, head covers, eye and respiratory protection, etc) is required. These PPE requirements will be indicated on the warning sign posted on the door of the animal's cage. Respiratory protection is provided by using properly fitted respirators approved by the National Institute of Occupational Safety and Health (NIOSH).[36] Surgical masks or nuisance dust masks do not meet the NIOSH definition of a respirator. NIOSH-approved respiratory protection systems are commonly used in BSL-3 laboratories and animal rooms when the respiratory hazard cannot be completely engineered out through the use of primary containment devices. Respirators used to filter particulates are classified into three series, corresponding to resistance to oil mist particles: (1) *N*, or least resistant, (2) *P*, or partially resistant, and (3) *R*, or resistant. They are further differentiated based on their efficiency at removal of 0.3 μm aerosol particles, similar to HEPA filters (95%, 99%, and 99.97% or –100).[37] Useful and comfortable negative-pressure respirators include disposable N-100 filtering face pieces with integral exhalation valves and tight-fitting, half-face, negative-pressure respirators fitted with N-100 particulate filters. These respirators have an assigned protection factor of 10, meaning there are 10-fold fewer particulates at the breathing zone inside the respirator than outside the respirator, providing the respirator is properly fitted and worn. A properly fitted and worn full-face piece, negative-pressure respirator has an assigned protection factor of 50 to 100 and also provides eye protection. All users of respirators must be enrolled in a respiratory protection program in accordance with the Occupational Safety and Health Administration (OSHA) Respiratory Protection Standard.[31] Users of tight-fitting respirators must be fit tested annually or when significant physical changes occur (weight gain or loss) using an approved qualitative or quantitative fit test. Wearers of tight-fitting respirators must not have facial hair that could interfere with the fit of the respirator, nor should eyeglasses interfere with the tight seal. Users of full-face, tight-fitting respirators who wear eyeglasses will need special optical inserts that may be worn inside the respirator face piece. Individuals fit tested for respirators must ensure that they only use respirators that they have been trained and certified to use during annual fit testing.

When working in a BSL-3 environment, such as a room housing infected animals in open cages or a necropsy room equipped with a downdraft table and an oscillating bone saw, greater respiratory protection might be needed. A NIOSH-approved PAPR with a loose-fitting hood or a tight-fitting full-face piece is often used and provides an assigned protection factor of 1,000. Benefits of wearing a loose-fitting hood include comfort, no requirement for fit testing, and amenability to use by individuals with facial hair. Reusable turbo blowers for PAPRs are powered by rechargeable batteries. The blowers may be equipped with N-100 particulate filters or with combination cartridges that incorporate a particulate filter with activated charcoal or other chemical absorbent for use in atmospheres of between 19.5% and 23.5% oxygen that have contaminated particulates and low levels of organic or other specified chemical vapors. The airflow in cubic feet per minute, with cartridges installed, must be checked with a flow gauge before each work session. Because there are no OSHA standards or end-of-service life indicators for particulate filters when used with infectious agents, institutes have to develop local criteria for determining when to replace particulate filters. As a complete protective ensemble, PAPRs with loose-fitting hoods may be worn in conjunction with Tyvek suits or long-sleeved scrub suits, gloves, laboratory socks, and shoes with shoe covers or over-boots. All NIOSH-approved respirators are approved as a complete system, so components cannot be switched between different manufacturers' products without negating the approval. For example, a NIOSH-approved PAPR system consists of the turbo blower unit, battery, belt, hose, filters or cartridges, and loose-fitting hood or tight-fitting face piece, all assembled and marketed by the manufacturer as a complete system. Only approved, compatible replacement components from the same manufacturer may be used with a given respiratory protection system.

To be approved to use a respirator, a user must be medically cleared based on a health history questionnaire and a pulmonary function test or other relevant medical examinations on a case-by-case basis; be enrolled in an employer-provided OSHA-compliant respiratory protection program[31]; receive initial and annual training on the use of the assigned respirator or additional training when a different type of respirator is assigned; and undergo annual fit testing for negative-pressure, tight-fitting respirators. Proper fit testing procedures are available in Appendix A of the OHSA Respiratory Protection Standard.[31]

In a class III BSC operation (BSL-4 cabinet laboratory), personnel must remove all personal clothing and undergarments and shoes. Complete laboratory clothing, including undergarments, pants, shirts, shoes, and gloves, is provided and worn by laboratory workers.[1] Workers wear nitrile or latex examination gloves for extra protection when working in class III BSCs, just in case the shoulder-length box gloves develop pinholes, punctures, or tears.

In BSL-4 protective suit laboratories and BSL-4 animal facilities, personnel must remove all personal clothing, including undergarments, socks, shoes, and jewelry. Personnel at USAMRIID may ask for an exemption for wedding bands, but only eyeglasses in addition to exempted wedding bands may be worn in the BSL-4 suites. Complete laboratory clothing, including undergarments, pants, shirts, jumpsuits, socks, and gloves, is provided for, and used by, laboratory workers. Workers don a fully encapsulating positive-pressure protective suit supported by an umbilical-supplied air system. It is common practice in BSL-4 laboratories for individuals to periodically verify PPES integrity prior to donning by taping the exhaust valves of the suit and inflating it to a set pressure point. This test is performed at USAMRIID at a minimum when the individuals change their gloves on a weekly basis, but practices vary at other BSL-4 facilities. In addition, annual pressure decay testing is conducted at USAMRIID on all PPES used in the BSL-4 laboratory. The suit can be fitted with integral protective over-boots or with legs terminating in soft booties. If a suit of the latter design is used, the worker dons protective over-boots inside the BSL-4 suit facility, after passing through an airlock equipped with a decontaminating chemical suit shower. When not in use, protective over-boots are stored inside the BSL-4 facility. As of this writing, PPES for use in a BSL-4 environment are not federally regulated by OSHA as level A chemical suits or as respirators, and such suits are not currently NIOSH approved. However, the compressor and filter system must provide minimum grade D breathing air to the PPES.[31]

Medical Surveillance

Medical surveillance, if indicated, may comprise baseline and periodic (usually annual) studies, including the following:

- complete medical history,
- urinalysis,
- hematology (complete blood count),
- serum chemistry panel,
- serum protective antibody titers for specific disease agents,
- physical examination, and
- ancillary studies.

Ancillary studies can include the following:

- periodic chest radiograph,
- periodic electrocardiogram,
- annual audiogram,
- annual visual acuity testing,
- annual evaluation of respiratory capacity, and
- mental fitness, neurological examinations, and random testing for illicit substance use (as needed).

An effective occupational health program benefits both the employee and the employer and may reduce time lost to injuries. This occupational health program will comply with OSHA and other applicable federal and state laws and regulations. Medical surveillance is a critical part of a comprehensive occupational health and safety program. An occupational health and safety program has the following objectives[38]:

- protect workers against health and safety hazards in the work environment;
- properly place workers according to their physical, mental, and emotional abilities;
- maintain a pleasant, healthy work environment;
- establish preplacement examinations;
- establish regular, periodic health examinations (medical surveillance);
- diagnose and treat occupational injuries, exposures, and diseases;
- consult with the worker's personal physician, with the worker's consent, regarding other related health problems;
- provide health education and counseling for workers;
- provide safety education for workers;
- identify hazardous situations or find the means to prevent or mitigate hazardous situations; and
- establish surveys and studies of the industrial environment to protect workers, their families, and the community.

Laboratory workers employed in a BSL-4 suit facility are enrolled in a medical surveillance program, and they should be medically evaluated for fitness to use a PPES. At USAMRIID, workers in the BSL-4 suit laboratories are enrolled in a hearing protection program. When the 8-hour, time-weighted average level is 85 dB (decibels) or greater, workers must be enrolled in an employer-provided hearing protection program

to comply with OSHA regulations.[39] The program requires employees to undergo initial baseline and annual surveillance audiometry, fitting, and training to use hearing protectors (ear plugs or muffs).

Personnel are required to receive initial familiarization training on how to wear the protective suit, as well as receive extensive, documented, tailored training provided by an assigned mentor before being considered proficient to work independently in BSL-4 containment. Currently, USAMRIID enrolls new personnel who plan on working in BSL-4 containment in a specialized 3-day training course teaching them the fundamentals of the BSL-4 environment, suit use, entry and exit procedures, movement and dexterity exercises, and emergency response. Once employees complete the basic training course, they are then mentored for a set period of time before they may apply for independent access to BSL-4. After demonstrating proficiency, laboratory workers can begin independent work in the BSL-4 containment suite.

During normal operations in the BSL-4 containment suite, workers may disconnect briefly from the breathing air supply to move about and then couple to an airline in a new location within the suite. One manufacturer advises that up to a 5-minute residual air supply may remain in the suit if there is an unanticipated loss or interruption of the breathing air supply.[40] In regular operations, it is prudent not to remain disconnected from the air supply for more than 2 or 3 minutes, because the carbon dioxide concentration and humidity level will quickly rise within the suit space. It is recommended as a best practice to remain connected to the air supply as much as possible when completing work tasks in BSL-4. Generally, the visor fogs ups before the carbon dioxide concentration builds up to a hazardous level, thus prompting the user to connect to the air supply expeditiously.

It is important that personnel are fit for the physical challenges of working in a BSL-4 PPES laboratory. An ongoing medical surveillance program ensures that, in the event of occupational exposure to an infectious agent or toxin, the medical needs of the worker will be met immediately. If a laboratory worker should become ill without obvious exposure to an agent, the individual will be assessed to determine whether the illness is related to an unknown laboratory exposure.

At USAMRIID, all potential biological exposures are assessed through the combined efforts of the Safety, Radiation, and Environment Division; personnel supervisors; and the Medical Division. All employees are instructed to notify the Safety, Radiation, and Environment Division of any mishaps occurring either inside or outside containment suites. For mishaps in the containment suites, all personnel involved in the incident are instructed to report to the Medical Division in the absence of a life-threatening emergency for an initial briefing. The initial briefing is conducted with the affected personnel, supervisors, a safety representative, and the competent medical authority. After the briefing, initial exposure and disease risk are determined and postexposure prophylaxis is administered (as determined by subject matter experts).[41,42] In the event of a medical emergency or potential exposure in BSL-4 containment, the Department of Defense currently has a memorandum of agreement with the Department of Health and Human Services for potential exposure monitoring, care, and treatment of inpatients enrolled as clinical research subjects. Local arrangements are made for laboratories outside the United States.

Protecting the Community and the Environment

Secondary barriers are the elements of laboratory facility design and construction that (*a*) contribute to protection of laboratory personnel, (*b*) provide a barrier to protect persons outside the laboratory, and (*c*) protect persons and animals in the community from infectious agents in the event of an accidental release within the laboratory.[1] Secondary barriers in BSL-3 containment facilities at USAMRIID include entry vestibules or personnel airlocks that feature two self-closing and lockable doors, clothes change rooms and shower facilities based on a risk assessment, and a hand-washing sink in each laboratory room. The sink is located near the room exit door and can be operated using foot pedals or knee or elbow paddles, or is automatically activated by an infrared sensor. Other secondary barriers include floor, wall, and ceiling finishes constructed for easy cleaning and decontamination; sealed penetrations in floors, walls, and ceilings; and sealable openings to facilitate decontamination. Laboratory furniture has waterproof and chemical-resistant bench tops, and chairs are covered with nonfabric material to permit easy decontamination. An autoclave is available in the facility. The facility is equipped with a ducted exhaust ventilation system that creates inward directional airflow from areas of lower potential hazard to areas of higher potential hazard (negative-pressure gradient) without recirculation of air or airflow reversals under failure scenarios.[1] To confirm inward airflow, a visual monitoring device (eg, a magnehelic differential pressure gauge [Dwyer Instruments, Michigan City, IN]; photohelic gauge [Dwyer Instruments, Michigan City, IN]; rodimeter; or "telltail") should be available at the laboratory entry.

In animal biosafety level 3 (ABSL-3) facilities, room fittings and ventilation should be in accordance with the Institute of Laboratory Animal Resources Commission on Life Sciences and National Research Council's *Guide for Care and Use of Laboratory Animals*[43] and

Biosafety in Microbiological and Biomedical Laboratories.[1] If the ABSL-3 facility has floor drains, the drain traps are always filled with an appropriate disinfectant. Additional environmental protection design features (enhancements) in BSL-3 laboratories and animal-holding spaces (including provision of personnel showers and effluent decontamination, HEPA filtration of exhaust air, and containment of piped services) may be indicated, depending on the nature of the infectious agents to be used (eg, arboviruses, highly pathogenic influenza viruses and high-consequence animal pathogens); the risk assessment or maximum credible event analysis of the site (eg, laboratory to be located in a highly populated urban center or in a remote region having a low-density population); and applicable federal, state, and local regulations.

Secondary barriers required in BSL-4 laboratories and ABSL-4 animal-holding spaces are all those specified for BSL-3 laboratories and ABSL-3 animal holding spaces, with additional provisions. Other required secondary barriers include a dedicated, non-recirculating ventilation system with supply and exhaust components balanced to ensure directional airflow from areas of lower potential hazard to areas of higher potential hazard. HEPA filtration of supply air and double HEPA filtration of exhaust air, with redundancy (backup exhaust duct with fan and in-line double HEPA filters), are also required, as is alarm and daily monitoring to prevent positive pressurization of the laboratory or animal-holding space. In large, complex operations, a supervisory control and data acquisition system (also known as a building automation system) may be installed to monitor and control room pressures automatically. An automatically starting emergency power source (usually a diesel-powered generator) is required as a minimum for the redundant exhaust ventilation systems, redundant life-support (breathing air) systems, alarms, lighting, entry and exit controls, and BSCs. Laboratories using PPES are required to have primary and backup breathing air compressors along with a secondary breathing air system capable of supporting the egress of all personnel in the BSL-4 suites in the event of a breathing air compressor failure. In practice, the freezers and other laboratory equipment (incubators and refrigerators) are generally also on circuits that can switch to emergency backup power. Other infrastructure elements that contribute to the secondary barrier include change rooms, personnel showers, effluent decontamination by a proven method (preferably heat treatment), and containment of piped services. Floor and sink drain traps must be kept filled with an appropriate disinfectant (one with proven efficacy for the microorganisms handled within the BSL-4 facility). An autoclave with two interlocked doors, with the outer door sealed to the outer wall (a so-called "bioseal"), is required at the containment barrier. The autoclave is automatically controlled so the outer door cannot be opened until a sterilization cycle has been completed. A dunk tank, fumigation chamber, or a ventilated equipment airlock is also provided so materials may pass into the containment area. Materials that cannot be steam sterilized may be safely decontaminated either through a fumigation cycle in a ventilated airlock or by passage through the chemical shower cycle or dunk tank and removed from the containment area. The walls, floors, and ceilings are constructed as a sealed internal shell (the containment envelope) capable of being decontaminated using a fumigant. Bench tops have seamless surfaces impervious to water, resistant to chemicals, and free of sharp edges. Appropriate electronic communications are provided between the BSL-4 containment area and the noncontainment area, which may include a telephone, facsimile, two-way radio, intercom, and a computer system on a local area network or wireless network. BSL-4 protective suit laboratories also have a dedicated area for storing suits and boots, and a double-door personnel airlock equipped with a chemical shower for surface decontamination of protective suits. Animal-holding rooms need to meet the standards specified in the *Guide for Care and Use of Laboratory Animals*.[43] Containment operational parameters are inspected and verified daily before work is initiated in the BSL-4 facility.

Solid and Liquid Waste Inactivation and Disposal

The US Environmental Protection Agency (EPA) defines antimicrobial pesticides as substances or mixtures of substances used to destroy or suppress the growth of harmful microorganisms (eg, bacteria, viruses, or fungi) on inanimate objects and surfaces. Public health antimicrobial products are intended to control microorganisms infectious to humans in any inanimate environment. These products include sterilizers (sporicides) and disinfectants.[44] Sterilizers (sporicides) are used to destroy or eliminate all forms of microbial life, including fungi, viruses, and all forms of bacteria and their spores. Sterilization is widely used in hospitals for infection control. Types of sterilizers include steam under pressure (autoclaves), dry-heat ovens, low-temperature gas (ethylene oxide), and liquid chemical sterilants. All types of sterilizers are also applicable for use in microbiological and biomedical laboratories. In laboratories, autoclaving is used to prepare sterile instruments, equipment, and microbiological nutrient media and to render microbiologically contaminated liquid and solid waste sterile before it

enters the waste disposal stream. Laboratory glassware is dried, sterilized, and depyrogenated (rendered free of endogenous pyrogens) in dry-heat ovens. Ethylene oxide sterilization is used to sterilize materials such as delicate instruments and laboratory notebooks, which cannot withstand steam sterilization, but is seldom used to sterilize solid waste. Liquid sterilants, used to sterilize delicate instruments by immersion and to sterilize impervious surfaces by surface application, can be added to suspensions of infectious materials to chemically inactivate them. Disinfectants, according to the EPA, are used on hard inanimate surfaces and objects to destroy or irreversibly inactivate infectious fungi and bacteria, but not necessarily their spores. The EPA divides disinfectant products into two major types: hospital and general use. Hospital disinfectants are most critical to infection control in hospitals and are used on medical and dental instruments and on hospital environmental surfaces. General disinfectants are products used in households, swimming pools, and water purifiers.

The decision about the type of biological inactivation required depends on a number of factors; the type of biological agent requiring inactivation along with whether the agent is present in a large amount of organic material can initially narrow the choice. In some cases where either large spills or large amounts of organic material are present, a detergent solution may be used prior to disinfectant to enhance efficacy at the site of cleanup. Other variables, including but not limited to pH, temperature, type of materials requiring biological inactivation (eg, neoprene, metals, or plastics), age of disinfectant, humidity, concentration, and contact time requirements can also affect the choice of inactivation method.

An example of a liquid sterilant-disinfectant is Alcide EXSPORE (Alcide Corporation, Redmond, WA) 4:1:1 base concentrate (1.52% sodium chlorite; EPA Registration No. 45631-3), which comes with a separate activator concentrate (9.5% lactic acid) as a set. This sterilant-disinfectant must be freshly prepared by diluting the base with water per the manufacturer's instructions before adding activator to generate chlorine dioxide.[45] The prepared sterilant-disinfectant should be used immediately and must be freshly prepared daily.

An example of a hospital disinfectant is Micro-Chem Plus (National Chemical Laboratories, Inc, Philadelphia, PA; EPA Registration No. 1839-95-2296), a proprietary mixture of two quaternary ammonium compounds and inert ingredients that is labeled to kill listed microorganisms (specified viruses, fungi, and nonspore-forming bacteria) when mixed at the rate of 2 ounces of the concentrated product per gallon of water.[46]

An example of a general disinfectant used at USAMRIID is Clorox Ultra Germicidal Bleach (The Clorox Company, Oakland, CA; 6.15% sodium hypochlorite [5.84% available chlorine]; EPA Registration No. 67619-8). When mixed at the rate of 12 ounces per gallon of water (5,000 ppm), it is labeled to kill listed microorganisms (specified viruses, fungi, and nonspore-forming bacteria).[47] Bleach is not registered by the EPA as a sterilant. During the subsequent cleaning and decontamination of spore-contaminated postal facilities after the 2001 anthrax-by-mail incidents, the EPA issued crisis exemptions on a case-by-case basis to use bleach for emergency decontamination subject to adherence with specified conditions of application (see http://www.epa.gov/pesticides/factsheets/chemicals/bleachfactsheet.htm). Bleach solutions (1:10 dilution) are now sold premixed, as standard 1:10 dilutions must be made fresh daily. Clorox disinfecting wipes (EPA Registration No. 5813-79) also come ready-made with an extended shelf life compared to fresh 1:10 solutions. Wipes may be used in the laboratory or in the field with the same efficacy as fresh daily 1:10 solutions.

In BSL-4 laboratories and in BSL-3 and ABSL-3 facilities, if indicated by the risk assessment, liquid effluent (laboratory sewage) must be inactivated by a proven process, generally heat treatment under pressure.[1] Effluent decontamination systems are available as six different types. Systems may be batch-based chemical systems using peracetic acid, sodium hypochlorite, quaternary ammonium compounds, or chlorine dioxide. Heat treatment effluent decontamination systems may be either continuous flow or batch process and can run at high temperature (>121°C) or sub-boiling temperatures. Batch process effluent decontamination systems models can also be designed to run as a heat treatment system augmented by chemicals depending on the demands of the laboratory. Solids suspended in the liquid waste are comminuted (finely ground). The effluent is heated to specified temperature and held at that temperature for a certain period of time. Then it is cooled, sampled for sterility testing, and released to a municipal or nonpublic sewer system. The time–temperature relationship for the selected process depends on the inactivation profile of the infectious microorganisms that could be present in the liquid waste. The current process at Fort Detrick holds the heated effluent at 132°C (270°F) for a minimum of 12 minutes, sufficient to inactivate fungal and bacterial spores. The standard liquid biowaste process used at the Canadian Science Centre for Human and Animal Health (Winnipeg, Manitoba, Canada) heats the effluent to 121°C (250°F) for a 30-minute holding time, but has the capability of achieving a temperature as high

as 141°C (286°F).[48] The standard process is sufficient to inactivate fungal and bacterial spores. The higher temperature is available, if needed, to inactivate prions (heat-resistant infectious proteins).[49]

Animal carcasses exposed to biological agents inside BSL-3 or BSL-4 laboratories require decontamination prior to removal from the containment suites. Per SOP, carcasses are bagged and decontaminated with the appropriate disinfectant prior to autoclaving. The autoclaved carcasses can be incinerated subsequently or disposed of in accordance with applicable local, state, and federal regulations. Animal carcasses can also be inactivated with an alkaline hydrolysis-based tissue digester. Digesters can be run either at boiling or sub-boiling temperatures using potassium hydroxide or sodium hydroxide alkali for a set cycle time. Cycle time depends on weight, composition, and surface area of animal carcasses, percent of alkali, temperature, and amount of water.[50–52]

After infectious materials have been inactivated by an appropriate method of sterilization or disinfection, they may be removed from the laboratory and disposed of in accordance with applicable federal, state, and local regulations. In the United States, disposal of several categories of solid waste (regulated medical waste, perceived medical waste, and pathological waste) is regulated at the state level. Many states have strict regulations that require that such waste be sterilized and rendered unrecognizable (by processes such as incineration, shredding, or grinding with steam sterilizing or irradiating) before final disposal in a sanitary landfill.

Standard and Special Microbiological Practices

Standard and special microbiological practices universal to all BSLs are as follows:

- The laboratory director limits or restricts access to the laboratory when experiments are in progress.
- A biohazard sign may be posted at the entrance of the BSL-1 laboratory if infectious agents are present or stored in the laboratory. A biohazard sign is posted at the entrance of BSL-2, BSL-3, and BSL-4 laboratories and animal rooms when infectious agents are present.
- Policies for the safe handling of sharps are instituted.
- All procedures are performed carefully to minimize the creation of aerosols.
- Work surfaces are decontaminated at least once daily and after any spill of viable material.
- All infectious waste is decontaminated by an approved process (eg, autoclaving before disposal).
- A pest (insect and rodent) control program must be in effect.
- Personnel wash their hands after handling viable materials, after removing gloves, and before leaving the laboratory.
- Eating, drinking, smoking, handling contact lenses, taking medication, and storing food for human consumption in the laboratory or animal-holding facility are not permitted. If contact lenses are worn in the laboratory or animal-holding area, goggles or a face shield should also be worn. Personnel should refrain from applying cosmetics or lip balm, chewing gum, and taking oral medications while in the laboratory or animal-holding facility.
- Mouth pipetting is prohibited. Only mechanical pipetting devices are to be used.

There are no special practices for the BSL-1 laboratory. The following special practices apply to BSL-2, BSL-3, and BSL-4 laboratories, as well as to ABSL-2, ABSL-3, and ABSL-4 animal-holding areas:

- Secure all laboratories registered for select agents and toxins.[53] Keep BSL-2 and BSL-3 laboratory room doors closed when working with infectious agents. Keep doors in BSL-4 laboratories and in ABSL-2, ABSL-3, and ABSL-4 animal-holding areas closed and locked.
- Only individuals advised of the potential hazards who meet specific entry requirements may enter the laboratory or animal-holding room.
- In ABSL-2, ABSL-3, and ABSL-4 animal-holding facilities, the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee approve special policies and procedures.
- Along with the biohazard sign, post the following information at the entrance to the laboratory or animal-holding room: the agents in use, the BSL, required vaccinations, any PPE required, the name and phone number of the principal investigator, and any procedures required to exit the laboratory or animal-holding room.
- At-risk individuals entering the laboratory or animal-holding room may receive appropriate vaccinations if available for the agents being handled or agents potentially present in the room.

- A tuberculosis skin test or other tuberculosis surveillance procedures are indicated on an annual basis if personnel are working with or around nonhuman primates.
- Describe biosafety procedures for BSL-2 and ABSL-2 facilities in SOPs. Describe biosafety procedures for BSL-3 and BSL-4 laboratories and ABSL-3 and ABSL-4 animal-holding facilities in a biological safety manual specific to the laboratory or animal-holding facility. Advise personnel of the specific hazards, require them to read and ensure they understand the manual, and make certain that they comply with it.
- The laboratory director must ensure that laboratory and support personnel receive appropriate initial training and annual training, and additional training on potential hazards in the laboratory or animal facility; precautions to take to prevent exposures; and procedures on evaluating potential exposures. The laboratory director is also responsible for ensuring that the previously described training is appropriately documented.
- Use caution with needles and syringes. In BSL-3 and BSL-4 laboratories and in ABSL-3 and ABSL-4 animal-holding facilities, use only needle-locking syringes or disposable syringe–needle systems in which the needle is integral to the syringe. Also consider tools that allow for one-handed recapping of syringe needles or systems without needles. Dispose of used sharps in conveniently located puncture-resistant containers.
- Place all potentially infectious materials in covered, leak-proof containers during collection, manipulation, storage, transport, or shipping. Place viable material to be removed from a class III BSC or a BSL-4 facility in an unbreakable, sealed primary container that is enclosed in an unbreakable, sealed secondary container. Pass this enclosed material through a chemical disinfectant dunk tank, fumigation chamber, or airlock with a chemical suit shower (in the case of a BSL-4 suit facility).
- Decontaminate work surfaces and laboratory equipment with an effective disinfectant routinely, after work with infectious materials is completed, and after any spills. Contaminated equipment must be appropriately decontaminated before repair or maintenance or packaging for transport.
- Immediately report to the laboratory director (supervisor) any spill or accident that results in exposure to infectious materials. Institute medical evaluation, surveillance, and treatment as appropriate and document this medical care in writing. In BSL-3 and BSL-4 containment facilities, develop and post spill procedures and conduct drills on an annual basis. Professional staff or other appropriately trained personnel must decontaminate, contain, and clean up any spill of infectious material. In BSL-4 containment, establish practical and effective protocols for emergency situations, including the evacuation of incapacitated staff.
- Animals and plants unrelated to the work conducted are not permitted in the laboratory.
- In BSL-3 and BSL-4 containment facilities, the laboratory director must ensure that all personnel are proficient in standard microbiological practices, laboratory-specific practices, and operations before they begin work with microorganisms.
- In BSL-3 and BSL-4 containment facilities, conduct open manipulations of infectious agents in BSCs or other primary containment devices. Conducting work in open vessels on the open bench is prohibited. Vessels with tight-fitting covers (gasketed caps, O-ring seals) should be used to hold viable cultures within water baths and shaking incubators. Use sealed rotors or centrifuge safety containers fitted with O-ring seals to contain centrifuge tubes. Use plastic-backed paper towels on nonperforated surfaces to facilitate cleanup. Use plastic vessels in place of glass vessels.
- In BSL-3 and BSL-4 containment facilities, autoclave or decontaminate all materials other than materials to be retained in a viable state before removing them.
- At BSL-4, maintain a physical or electronic log of all personnel, with the time of each person's laboratory entry and exit recorded. This requirement also applies to all personnel who have access to areas in which select agents and toxins are used or stored.[53]
- In BSL-4 containment (and in BSL-3 containment, if indicated by risk assessment, site-specific conditions, or applicable regulations), enter and exit the laboratory only through the clothing change and shower rooms. Remove and leave personal clothing in the outer change room. Change completely into laboratory clothing. On exiting the laboratory, remove and leave all laboratory clothing in the inner change room. Take a decontaminating (soap and water) personal wet shower for a minimum of 3 minutes on exit from the laboratory.

Autoclave soiled laboratory clothing before laundering. Use the equipment airlock to enter or exit the laboratory only in an emergency.
- Bring supplies and materials into the BSL-4 facility through the double-door autoclave, fumigation chamber, or equipment airlock, which is decontaminated before and after each use. Secure the airlock outer door before the inner door is opened. Secure the airlock inner door after materials are brought into the facility.
- In BSL-4 containment, institutes are required to establish a system to report laboratory accidents and exposures, employee absenteeism, and medical surveillance of a potential laboratory-acquired illness.[1]
- Make available a facility for quarantine, isolation, and medical care of personnel who work in BSL-4 containment and who are affected with a potential or known laboratory-acquired illness.

ROLE OF MANAGEMENT IN A BIOSAFETY PROGRAM

Management must consider safety a top priority and, on a daily basis, work closely with and support safety personnel. Although management must provide a biosafety program as well as engineering features and equipment designed to reduce the risks associated with the research conducted at the institute, safety is also an individual responsibility. To illustrate this point (Figure 30-1), consider the mission or purpose of an institute as the hub of a wheel. All personnel, regardless of education, experience, or job description, are the spokes of the wheel and must be reminded regularly of the importance of their contributions to an institute. If one (or more) of the spokes is not functioning as designed, the wheel does not operate smoothly. Consequently, it takes longer to meet not only personal goals and objectives, but also institute goals and objectives. All personnel (each spoke of the wheel) in an institute must be considered important, regardless of their perceived contributions.

The goals of a biosafety program include the following: (*a*) prevention of injury, infection, and death of employees and the public; (*b*) prevention of environmental contamination; (*c*) conformance to prudent biosafety practices; and (*d*) compliance with federal, state, and local regulations and guidelines. The ultimate objective of these goals is to keep everyone healthy while supporting productive research. Appropriate personnel training is paramount. Both initial and refresher personnel training must address the institutional biological safety program and the elements of biosafety. Training can be conducted as a discussion rather than as a formal lecture to promote audience participation. This technique allows individuals to have ownership over policies through an integrated program of safety engineering, vaccination, health surveillance, and medical management of illness. Risk encompasses awareness, assessment (or evaluation), mitigation, and management of the risk. Communication is a fundamental part of risk assessment and training. The US government has developed a 5-step risk management process (Figure 30-2).[54] The five sequential steps of the risk management process include the following:



Figure 30-1. Institute personnel are depicted as the spokes of a wheel that work together to accomplish a common mission. *maintenance staff

1. **Identify hazards**. What is the hazard?
2. **Assess hazards**. What is the danger of this hazard?
3. **Develop controls and make risk decision**. What controls can be used to remove this hazard, or make a decision to accept some risk?
4. **Implement controls**. Controls developed for the risk are implemented (or put into operation or practice).
5. **Supervise and evaluate**. After a period of evaluation as new data becomes available, the controls implemented are reviewed to determine whether they were adequate, or if additional controls must be added.

The philosophy of a biosafety program is based on an early estimation of risk, followed by application of appropriate containment and protective measures.



Figure 30-2. Five steps of the risk management process. Adapted from: US Army Safety Center, Fort Detrick, MD.

It is very important to investigate and review safety incidents at the institute because presentation of this data will heighten the awareness of individuals that accidents do happen despite safeguards.

Laboratory Safety Audits

An audit is a methodical examination and review. In the present context, it is a systematic, critical review of laboratory safety features and procedures. The terms "survey" (comprehensive view) and "inspection" (a critical appraisal, description of some obvious hazards and how safety personnel try to minimize the risk of these hazards, an official examination, or checking or testing against established standards) are often used interchangeably with the term "audit." Safety personnel must emphasize that their role is to try to identify hazards, conduct risk assessments, develop risk management strategies, and evaluate the effectiveness of those strategies over time while minimizing impact on the research. Safety personnel must actively engage with and seek the help of all administrative and laboratory personnel in hazard identification. It is important for safety personnel to remain actively engaged with laboratory personnel outside of laboratory audits to minimize potential negative associations that may be encountered with inspections. It must be understood that a safety department cannot provide absolute safety, but strives to provide reasonable safety. Safety personnel advise, guide, provide limited training, and implement institute and regulatory policies (in conjunction with the institutional biosafety committee). The safety department, with continued support from management and all facility personnel, can minimize the risk of hazards by implementing institute and regulatory guidelines. During the laboratory safety audit, safety practices and equipment are evaluated. General safety, life safety, biological safety, chemical hygiene, and radiation safety are topics covered in a typical laboratory safety audit. Laboratory audits should be scheduled on a regular basis and may be announced or unannounced.

Self-audits of required safety practices provide a measure for achieving compliance with safety rules and regulations.[55] Designated safety specialists can conduct regular safety audits at quarterly intervals, accompanied by the laboratory supervisor and a facilities management representative. Deficiencies can be pointed out during the audit. Later, a written report with suggestions for corrective action may be sent to the laboratory supervisor. The supervisor reports progress on remediation to the safety specialist within a mutually agreed on, fixed-time period. Safety personnel should follow up on any deficiencies noted during a laboratory audit periodically to ensure laboratory personnel have taken the appropriate corrective actions. Support from higher management is essential for an audit to have the desired effect of improving employee safety, as well as instituting compliance with applicable regulations.[56]

Use of a checklist ensures a systematic, standardized audit, thus reducing the chance of missing critical items. Citing the pertinent requirement or applicable regulation on the checklist provides a ready reference and justification for each item listed on the checklist. Within the overall laboratory safety audit, the following list of biosafety elements should be covered[57]:

- autoclave repair and operational records where applicable,
- proper use of PPE,
- appropriate laboratory clothing,
- no food or drink in the laboratory,
- proper use of sharps and sharps disposal containers,
- decontamination of infectious materials before disposal,
- proper disposal of laboratory waste,
- proper signage (laboratory, equipment, materiel),
- current certification of BSCs and fume hoods, and
- use of in-line HEPA filters on laboratory vacuum outlets where applicable.

Additional biosafety elements audited at USAMRIID include: (*a*) weekly flushing floor and sink drains and recording the action in a drain flush log; (*b*) flushing the eyewash weekly and recording the action in an eyewash flush log; (*c*) testing (flushing and measuring the flow rate) the emergency deluge shower at least weekly and recording the action in an

emergency shower test log; (*d*) recording during the audit differential pressures for laboratory rooms as displayed on the magnehelic and photohelic gauges; (*e*) checking documentation that emergency communication devices have been tested at least monthly; (*f*) testing and recording during the audit operating status of alarms, emergency lights, and emergency exit lights; and (*g*) spot checking laboratory SOPs, laboratory biosafety manuals, and laboratory personnel training records.

Four events that warrant conducting a formal, unscheduled audit of a laboratory include the following[57]:

1. accident or injury in the workplace,
2. follow-up to implementation of new biosafety regulations or procedures,
3. a new funding source requesting documentation of workplace safety, and
4. new infectious agents proposed for use in the laboratory.

An important time for evaluation of biosafety SOPs may be before a major outside organization or agency conducts a site visit.[57] Two examples of organizations conducting site visits are the Joint Commission on Accreditation of Healthcare Organizations and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. Examples of agencies that conduct inspections of laboratories registered for select agents are the US Centers for Disease Control and Prevention and US Department of Agriculture Animal and Plant Health Inspection Service Select Agent Program Laboratory Inspection Programs. Laboratories that do not work on select agents may be subject to a US Department of Agriculture inspection for specific biological agents or an NIH Office of Biotechnology Activities audit if they have a functioning Institutional Biosafety Committee.[58] For subordinate laboratories of the US Army Medical Research and Materiel Command, safety office personnel conduct periodic safety site assistance visits.[30] For Department of Defense (DoD) research, development, test, and evaluation (RDTE) laboratories, the director of Army safety conducts biological defense safety evaluation site visits.[30]

In DoD RDTE facilities, health and safety professionals must conduct internal inspections (audits) of BSL-1 and BSL-2 laboratories at least quarterly and must conduct internal inspections of BSL-3 and BSL-4 laboratories at least monthly.[34] Inspections must be documented, deviations from safe practices recorded, and recommended corrective actions taken. If deviations are life threatening, access to the laboratory area is restricted until corrective actions have been taken. New RDTE efforts involving biological agents must be evaluated and inspected before startup. Any Department of the Army headquarters agency can recommend special studies or reviews when (*a*) conditions or practices that may affect safety have changed, (*b*) major system modifications to facility design and physical configuration are made, and (*c*) safety, health, and environmental protection standards and requirements have changed significantly.[30] Safety officials maintain safety inspection records for 3 years, and they review records annually to note trends that require corrective actions.[30] Laboratory supervisors review their work areas at least weekly and take any needed corrective actions promptly.

At USAMRIID, safety professionals assigned to the Office of Safety, Radiation and Environment conduct semiannual comprehensive inspections of BSL-1 and BSL-2 and quarterly inspections of BSL-3 and BSL-4 laboratories to identify potential problems. These quarterly inspections augment the monthly inspections conducted by laboratory suite supervisors or their designees. Inspections, which may be announced or unannounced, include coverage of general safety practices and safety practices specific to a particular BSL.[57]

Biological Defense Research Program Laboratories

All laboratories involved in DoD RDTE operations must comply with the Department of the Army Pamphlet, *Safety Standards for Microbiological and Biomedical Laboratories*.[34] These regulations specify safety policy, responsibilities, and procedures for military and contract laboratories conducting operations at BSL-2, BSL-3, and BSL-4 in support of the US military biological defense program. The DoD Biological Surety (Biosurety) Program is a new program implemented in DoD biological defense RDTE laboratories that use DoD-provided biological agents.[59] This biosurety program is patterned after existing nuclear and chemical surety programs, and its purpose is to ensure the safe and secure use of biological agents. The program encompasses physical security, biological safety, biological agent accountability, and personal reliability as measures to prevent unauthorized access to agents of bioterrorism (select agents).[59,60]

Laboratory Animal Care and Use Program

Federal animal welfare regulations[61–63] from the US Department of Agriculture Animal and Plant Health Inspection Service, state and local laws, and the Public Health Service Policy on Humane Care and Use of Animals[64] regulate the care and use of laboratory animals

used in research. Many of the applicable regulations and policies are summarized in the *Guide for the Care and Use of Laboratory Animals*.[43] The responsible administrative official at each institution using laboratory research animals must appoint an Institutional Animal Care and Use Committee representative to oversee and evaluate the institution's animal program, procedures, and facilities on a semiannual basis to ensure they are consistent with the animal welfare regulations, Public Health Service policy (for those institutions that receive NIH funding), and recommendations specified in the guide.[43] The guide covers many aspects of an institutional animal care and use program, including the following:

- policies and responsibilities,
- monitoring care and use of animals,
- veterinary care,
- qualifications and training of personnel who work with animals, and
- occupational health and safety of personnel working with animals, physical facilities, and animal husbandry.

Under the heading of occupational health and safety, critical topics in an effective animal care and use program include the following:

- hazard identification and risk assessment;
- personnel training, hygiene, safe facilities, and procedures;
- health monitoring;
- animal experimentation involving biological and other hazardous agents;
- use of PPE;
- medical evaluation; and
- preventive medicine for personnel working with animals.

A voluntary program exists for the assessment and accreditation of institutional animal care and use programs. At the request of a given institution, the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) will send laboratory animal technical experts to the institution to conduct a site visit and evaluate all aspects of an institution's animal care and use program. If all aspects of the program meet the high standards of AAALAC, the institution may be granted the coveted designation "AAALAC accredited," which is effective for 3 years. Triennial renewals require a complete, comprehensive reassessment of an institution's animal care and use program. Accreditation by AAALAC is mandatory for DoD organizations and facilities maintaining animals for use in DoD programs.[64]

THE BIOSAFETY PROFESSION

Many biological safety professionals begin their careers as bench scientists in the biological sciences, particularly microbiology, or as professionals in medicine or the allied health sciences, and subsequently transfer into the biological safety field to work as biological safety officers, occupational health and safety managers or specialists, or in closely related positions. With the quickening tempo of biological defense research and the establishment of new, high (BSL-3 or BSL-4) biocontainment laboratories, the demand for competent biological safety professionals is increasing. Academic institutions and government agencies are beginning to recognize the need to establish didactic and practical training opportunities in biological safety. For example, the Division of Occupational Health and Safety and the National Institute of Allergy and Infectious Diseases of the NIH have jointly established a National Biosafety and Biocontainment Training Program offering 2-year postbaccalaureate and postdoctoral fellowships at the NIH campus in Bethesda, Maryland. This program specifically trains fellows to support BSL-3 and BSL-4 research environments by acquiring the necessary knowledge and skills to meet scientific, regulatory, biocontainment, biosafety, engineering, communications, management, and public-relations challenges associated with conducting research in such facilities.[65] Education is carried out through extensive mentorship and training in pertinent safety and regulatory guidelines within the 27 institutes and centers at the NIH-Bethesda campus. Second-year fellows then apply their knowledge through a series of developmental assignments at external facilities outside the NIH system to better develop a well-rounded understanding of prudent safety practices that they may apply after departing the fellowship. Examples of academic fellowship programs include the biosafety fellowship program at Washington University School of Medicine in St Louis, Missouri, or the 1-year internship program at the Great Lakes Regional Center for Excellence at the University of Chicago.

Credentialing biological safety professionals is not currently mandated or regulated. A formal, voluntary credentialing process exists to enable biological safety professionals to meet minimum set standards of expertise and proficiency. The American Biological Safety Association (ABSA), the national organization of biological safety professionals, has established two levels of credentialing: (1) the Registered Biosafety Professional (RBP) and (2) the Certified Biological

Safety Professional (CBSP). The RBP is an individual with a documented university education or specialized training in relevant biological safety disciplines who has submitted an application and has been found to be eligible for registration by the ABSA RBP Evaluation Review Panel.[66] The RBP has sufficient understanding of cell biology, pathogenic microbiology, molecular genetics, host immune responses, and concepts of infectious agent transmission to enable the RBP to apply safeguards when working with biohazardous materials.

The CBSP is an individual who has a combination of documented university education, specialized training, and experience in relevant biological safety disciplines, and has further demonstrated knowledge and proficiency by passing the Specialist Microbiologist in Biological Safety Microbiology examination administered by the National Registry of Certified Microbiologists of the American Society for Microbiology. Every 5 years, qualification as a specialist microbiologist may be renewed by submitting to the National Registry of Microbiologists evidence of acceptable continuing education credits or by retaking and passing the examination. The CBSP also participates in a certification maintenance program administered by ABSA in which the individual submits a certain number of acceptable certification maintenance points every 5 years to maintain certification.

SUMMARY

A successful biosafety program is based on an early estimation of risk and application of appropriate containment and protective measures. It is important to review safety incidents that occur in the institute, because these data will heighten individual awareness that accidents do happen despite implementing safeguards. The goals of a biosafety program are to:

- facilitate safe, productive research,
- prevent environmental contamination,
- conform to prudent biosafety practices, and
- comply with federal, state, local, and institutional regulations and guidelines.

To achieve the goals of the biosafety program, information pertaining to the program must be conveyed to the workforce, along with how it benefits the workforce. Presentation of concepts must be expressed in understandable terms. Initial and refresher training of personnel must address elements of biosafety and the institute's biological safety program. To promote audience attentiveness, participation, and retention of information, training is best conducted in an informal discussion format. Training success is gauged by how well the workforce collectively internalizes the biosafety program, as evaluated within the overall context of a positive safety culture that permeates all work attitudes and operations. Elements of a positive safety culture include the following[34]:

- applying (regularly) safety practices and using safety terms in the workplace;
- including safety practices in the employee's job description and performance appraisals;
- specifying and monitoring safe behaviors in the workplace;
- providing tangible rewards for promoting safety;
- articulating safety concerns in interactions with management, peers, and subordinates;
- emphasizing safety procedures when starting new tasks;
- briefing employees on safety procedures and the consequences of ignoring safety practices or engaging in unsafe behaviors;
- observing, reporting, and correcting hazards promptly;
- keeping staff up to date on regulatory and institutional changes; and
- using PPE appropriately (always).

Management must consider safety a top priority and work closely on a daily basis with safety professionals, who need their support on policies to be implemented. Management must provide a safety program, engineering features, and equipment designed to reduce research-associated risks in the institute. Biosafety professionals strive to provide reasonable assurance of biological safety, but cannot guarantee absolute safety. In the end, the success of the safety program depends on the employees themselves. Safety is as much an individual responsibility as any other assigned performance objective.

Acknowledgments

Opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the US Army and Alliance Biosciences. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals, and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*.[43] The facility where this research was conducted is fully accredited by AAALAC International.

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Chapter 31

BIOLOGICAL SURETY

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INTRODUCTION

BIOLOGICAL SURETY
- **Control of Biological Select Agents and Toxins**
- **Registration for Possession, Use, and Transfer of Biological Select Agents and Toxins**
- **Security Risk Assessment**
- **Biological Select Agents and Toxins Inventory and Accountability**
- **Centralized Management of Long-Term Biological Select Agents and Toxins**
- **Biological Select Agents and Toxins Inventory Audits**
- **Biological Select Agent and Toxin Transfers**
- **Reporting Theft, Loss, or Release of Biological Select Agents and Toxins**
- **Identifying Select Agents and Toxins**
- **Restricted Experiments**
- **Biosafety**
- **Personnel Reliability**
- **Biosecurity**
- **Incident Response and Emergency Management**

SUMMARY

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INTRODUCTION

Evidence of human engagement in chemical and biological warfare to terrorize individuals or opposing armies and concurrent efforts to reduce these threats date back to the dawn of civilization. Some of the more prominent reports of possible biological warfare from the past millennium include the poisoning of enemy water wells with rye ergot fungus, a hallucinating agent, by the Assyrians; the use of hellebore roots to poison the drinking water of Kirrha by Solon of Athens (600 BCE); the use of poison arrows dipped in gangrene- and tetanus-causing agents by the Scythian archers of the Trojan war (400 BCE); tossing of venomous snakes onto the opponent ships of Pergamus by Hannibal at Eurymedon (190 BCE); hurling decomposing human bodies into enemy water wells by Emperor Barbarossa at the battle of Tortona (1155); catapulting the cadavers of plague victims over the city walls of Caffa (now Feodosia, Ukraine) by the Tartars (1346); distributing blankets and handkerchiefs from smallpox-infected patients to Native Americans by the British troops (1763); and sale of clothing from yellow fever and smallpox-infected patients by Confederate soldiers to unsuspecting Union troops during the American Civil War.[1-5] Causative agents were linked to infectious diseases by 19th-century scientists Louis Pasteur and Robert Koch. Advances in the field of microbiology soon led to the isolation of microbial agents from diseased humans and animals. Moreover, the development of *in vitro* methods to grow these pathogens in large scale gave those interested in biological weapons a new perspective in selecting an agent based on its ability to cause fear, disease, and mass casualties.

Recognizing the destructive powers of war, especially the devastation caused by chemical and biological weapons, developed nations of the world have attempted to establish international rules of engagement by drafting treaties and declarations that primarily focused on disarmament, laws of war, and war crimes (Table 31-1). The 1st International Peace Conference in 1899 at Hague, The Netherlands, produced the Prohibition of the Use of Projectiles with the Sole Object to Spread Asphyxiating Poisonous Gases.[6] Ratified by all major powers except the United States of America, this declaration states that in any war between signatory powers, the parties will abstain from using projectiles, "the sole object of which is the diffusion of asphyxiating or deleterious gases." The 2nd International Peace Conference held in 1907 prohibited the use of poisons and weapons with poisons.[7] The major accomplishment of these peace conferences was the establishment of an international court for mandatory arbitration and dispute settlement between nations.

Despite the declaration prohibiting projectiles that spread poisonous gases, biological weapons were not unequivocally prohibited. The advent of World War I (WWI) led to rapid progression of chemical and biological weapons, particularly those that were developed and used by the German Army. Various chemical weapons were used extensively during WWI primarily to demoralize, injure, and kill entrenched enemies indiscriminately. These ranged from disabling tear gas to deadly phosgene and chlorine gases. Due to the widespread use of chemical weapons and rapid development of high-explosive agents during this war, WWI is often referred to as "The Chemists' War." With advances in the understanding of bacterial agents during the 19th century, the German Army launched a massive biological weapons campaign against the Allied Forces during WWI. However, instead of targeting humans, they concentrated on infecting livestock (horses and mules) with *Bacillus anthracis* and *Burkholderia mallei.* Several animals died from these infections, but these biological tactics failed to match the success of the chemical warfare efforts.[8]

After the end of WWI and with no lasting peace in sight, the Biological Weapons Convention developed the "Protocol for the Prohibition of the Use in War of Asphyxiating, Poison or Other Gases and the Bacte-

TABLE 31-1

TIMELINE OF INTERNATIONAL RULES AND TREATIES TO LIMIT OR BAN CHEMICAL AND BIOLOGICAL WEAPONS USE

Year	Significant Event
1899	1st International Peace Conference Prohibition of the use of projectiles to spread asphyxiating poisonous gases
1907	2nd International Peace Conference Prohibition of the use of poisons and weapons with poisons
1925	The Geneva Protocol Prohibition of germ (biological) and chemical warfare
1972	Biological Weapons Convention Prohibition of development, production, and stockpiling of biological weapons
1986	The Second Review Conference Establishment of confidence building measures

riological Methods of Warfare," signed in 1925 at Geneva, Switzerland, as an extension of the international peace conferences of 1899 and 1907. Also known as the "Geneva Protocol," this treaty permanently bans the use of all forms of chemical and biological warfare. However, it did not prohibit the use of biological or chemical agents for research and development, storage, and transfer. Many countries that signed on to the Geneva Protocol retained the right to retaliate against biological or chemical weapon attacks with their own arsenals. Treaties, declarations, and protocols produced by the international community continued to lack robust verification methods, leading to distrust among nations and reinvigoration of chemical and biological weapons programs prior to World War II (WWII). Several countries initiated biological warfare programs between the World Wars. The first scientifically informed use of biological agents as weapons began when the Japanese military conducted human experimentation with several infectious agents during combat, targeting military personnel and civilians in Manchuria and China.[1,2,9] During WWII, many countries, including the United States, Canada, United Kingdom, Germany, Japan, and the Soviet Union had active bioweapons programs with stockpiles of military significance. The Japanese military used biological weapons, killing tens of thousands of civilians and military.[1,2,9–12]

In 1972, US President Richard M Nixon made the decision to abandon biological weapons research and signed the Biological Weapons Convention, the first multilateral disarmament treaty banning development, production, and stockpiling of biological weapons. The US destroyed all biological weapon stockpiles and made the facilities that produced these weapons inoperable. Participant nations in the 2nd Review Conference in 1986 agreed to implement a number of confidence-building measures to prevent ambiguities, doubts, and suspicions and to improve international collaboration toward peaceful biological research.[13]

In 1995, an extremist microbiologist was arrested for obtaining *Yersinia pestis* by mail order in the United States. Concern about the ease with which disease-causing agents could be obtained led the US Congress to pass the Antiterrorism and Effective Death Penalty Act of 1996.[14] This act directed the US Department of Health and Human Services (HHS) to establish: (*a*) a list of biological agents and toxins ("select agents") that pose significant threat to public health and safety; (*b*) procedures for regulating the transfer of these agents; and (*c*) training requirements for entities working with these agents. HHS delegated this authority to the Centers for Disease Control and Prevention (CDC) to establish the Laboratory Registration and Select Agent Transfer Program in 1996. Congress significantly increased the oversight of biological select agents and toxins (BSAT) following the anthrax attacks of 2001 by passing the USA PATRIOT Act (Uniting and Strengthening America by Providing Appropriate Tools Required to Intercept and Obstruct Terrorism Act of 2001),[15] which restricted access to BSAT, and the Bioterrorism Act (Public Health Security and Bioterrorism Preparedness and Response Act of 2002),[16] which included increased safeguards, security measures, and oversight of the possession and use of BSAT. The Bioterrorism Act also granted similar regulatory authority to the US Department of Agriculture (USDA) over select agents that pose severe threat to animal and plant health or products.[17] This led to the establishment of the Federal Select Agent Program (FSAP).

The FSAP consists of the CDC Division of Select Agents and Toxins (CDC-DSAT) and the Animal and Plant Health Inspection Services (APHIS) Agricultural Select Agent Program that oversee the possession, use, transfer, and destruction of BSAT that has the potential to pose severe threat to public, animal, or plant health or to animal or plant products within the United States. This chapter details the key concepts of the FSAP and US Department of the Army's (DA's) Biological Surety Program (BSP) and highlights how implementation protects the worker, the community, and the environment.

BIOLOGICAL SURETY

Biological surety, or "biosurety," is a Department of Defense (DoD) program for commanders and directors to implement and monitor judicious application of core principles pertaining to control of BSAT, biosafety and occupational health, personnel reliability, biosecurity, and emergency response in all military laboratories involved in developing medical countermeasures to BSAT for service members and the public. The principles of safety, security, agent accountability, personnel reliability, and incident response plans formulated by chemical and nuclear surety programs were instrumental during the development of the DA's biological surety regulations.[18] Certain infectious agents and toxins, designated as BSAT, have the potential to pose a severe threat to public health and safety, animal or plant health, or animal or plant products, and their possession, use, and transfer are regulated by the HHS and the USDA under the Select Agent Regulations. In addition, research involving recombinant or synthetic nucleic acid molecules,

including the creation and use of organisms and viruses containing recombinant or synthetic nucleic acid molecules, is regulated by National Institutes of Health (NIH) Office of Biotechnology Activities. The intent of the DoD BSP is to properly safeguard BSAT that is in the possession or custody of DoD facilities against theft, loss, diversion, or unauthorized access or use, and to ensure that operations involving such agents are conducted in a safe, secure, and reliable manner per regulatory requirements.

The CDC-DSAT and APHIS Agriculture Select Agent Services monitor compliance of registered entities to HHS- and USDA-published final rules, outlined in 42 CFR Part 73,[19] 7 CFR Part 331,[20] and 9 CFR Part 121.[21] One of the key components of the BSP that was unique to the DoD is the Biological Personnel Reliability Program (BPRP), which ensures that individuals with access to BSAT meet high standards of reliability and suitability. Recent updates to FSAP regulations require individuals with access to Tier 1 BSAT (Exhibit 31-1) be enrolled in a "suitability" program similar to the DA's BPRP program. With this change, the FSAP and the BSP correspondingly enhance the safety of individuals working with BSAT, protect and safeguard communities with biocontainment laboratories, and monitor the security of BSAT in entities registered and authorized to work with these agents and toxins (Figure 31-1).

EXHIBIT 31-1

LIST OF TIER 1 BIOLOGICAL SELECT AGENTS AND TOXINS

Botulinum neurotoxins
Botulinum neurotoxin producing species of *Clostridium*
Ebola virus
Francisella tularensis
Marburg virus
Variola major virus (smallpox virus)
Variola minor virus (alastrim)
Yersinia pestis
Bacillus anthracis
Burkholderia mallei
Burkholderia pseudomallei
Foot-and-mouth disease virus
Rinderpest virus

Reproduced from: Centers for Disease Control and Prevention; Animal and Plant Health Inspection Services. List of Select Agents and Toxins. 12 September 2013. http://www.selectagents.gov/Select%20Agents%20and%20Toxins%20List.html. Accessed June 25, 2014.

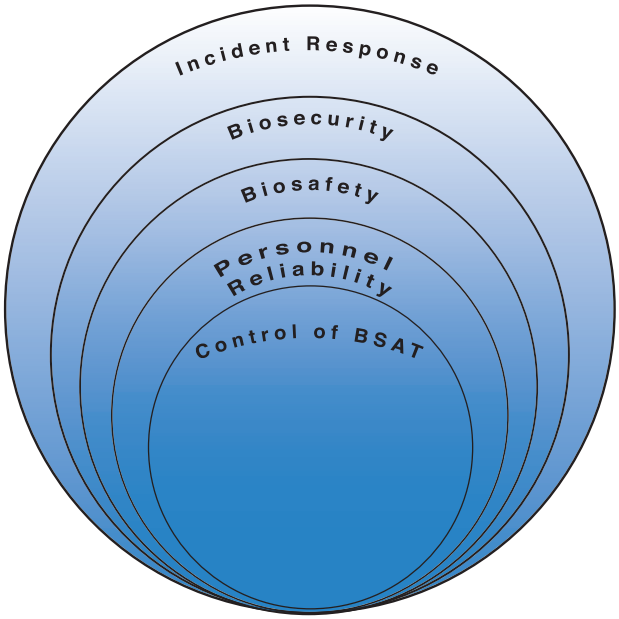


Figure 31-1. Key elements of the Federal Select Agent Program and Biological Surety Program.

Control of Biological Select Agents and Toxins

In accordance with 42 CFR Part 73,[19] the CDC-DSAT regulates agents and toxins that pose a severe threat to public health and safety. The APHIS Agriculture Select Agent Services regulates biological agents that pose a significant threat to plant and plant products in accordance with 7 CFR Part 331.[20] Agents that cause severe threat to humans, animals, and animal products are known as the "overlap agents" and are regulated by the CDC-DSAT and APHIS Agriculture Select Agent Services in accordance with 9 CFR Part 121.[21] In 2010, US President Barack Obama, through Executive Order 13546[22] directed HHS and USDA to: (*a*) designate a subset of BSAT (Tier 1,[23] see Exhibit 31-1) that presents the greatest risk of deliberate misuse with the most significant potential to cause mass casualties or devastating effects to the economy, critical infrastructure, or public confidence; (*b*) explore options for graded protection of Tier 1 BSAT to permit tailored risk management practices based on relevant contextual factors; and (*c*) consider reducing the overall number of agents and toxins on the select agents list. Federal BSAT regulations (42 CFR Part 73, 7 CFR Part 331, and 9 CFR Part 121) have been revised in accordance with Executive Order 13546.[20–22]

The FSAP mandates the appointment of a responsible official (RO) and an alternate responsible official (ARO) within each registered entity to monitor

compliance with the regulations governing select agents and toxins (SATs). Entities are authorized to appoint multiple AROs. The RO is granted authority and control to ensure compliance with FSAP regulations. In the absence of the RO, the ARO monitors entity compliance to FSAP regulations. In the DoD, a unit commander with a mission to conduct BSAT work (eg, development of diagnostics, medical countermeasures, etc) appoints an RO to monitor compliance of the entity to DoD, Army, federal, state, and local regulations governing BSAT. Regulatory oversight on entities that have a need to possess, use, and transfer BSAT is initiated by submission of various CDC APHIS forms that are specific for each regulatory component (Exhibit 31-2).[24]

Registration for Possession, Use, and Transfer of Biological Select Agents and Toxins

The FSAP requires all individuals, laboratories, and entities to register for possession, use, and transfer of BSAT. The first step in this process involves providing information through the completion of APHIS/CDC Form 1, Registration for Possession, Use, and Transfer of Select Agents and Toxins;[25] as described in 7 CFR 331,[20] 9 CFR 121,[21] and 42 CFR 73.[19] This form consists of several sections targeted to provide the regulatory agency with critical information on the biocontainment facility, safety, security, personnel, training, and research plans using SAT.

The entity is physically inspected by the FSAP following submission of the completed APHIS/CDC Form 1. The primary focus of this inspection is compliance with applicable federal regulations governing BSAT (7 CFR 331,[20] 9 CFR 121,[21] and 42 CFR 73[19]). During this visit, the inspectors verify the information provided in the submitted APHIS/CDC Form 1; evaluate personnel training, including mentorship programs; conduct interviews of personnel to identify issues related to biosafety, biosecurity, and training programs; check the engineering controls supporting the containment suites; and corroborate the commissioning or service records of all supporting machinery, including air-handling units, breathing-air systems, validation data for autoclaves, and all inactivation procedures to ensure that proper parameters are met and the methods used are determined to be efficacious with respect to producing nonviable waste. Ideally, entity registration is granted for 3 years after all inspection observations are satisfactorily resolved. However, a "conditional" registration may be granted under special circumstances (eg, during the interim when the entity needs to be operational to generate the data to satisfy a requirement). The FSAP inspectors ensure that the workers, communities, and the environment are not harmed by the operation of a containment or high containment laboratory.

APHIS/CDC Form 1 is also used to request changes to an approved registration. The entity must submit a letter to the FSAP requesting amendment to its registration and furnish the revised sections of the APHIS/CDC Form 1 related to the modifications. Most common amendments to registration involve addition and removal of personnel, name changes, addition or removal of agents or toxins, and changes in statement of work, including changes in project design, agent strains, animal models, modes of agent administration, and new laboratory projects.

EXHIBIT 31-2

ANIMAL AND PLANT HEALTH INSPECTION SERVICE/CENTERS FOR DISEASE CONTROL AND PREVENTION FORMS

- APHIS/CDC Form 1: Application for Registration for Possession, Use, and Transfer of Select Agents and Toxins
- APHIS/CDC Form 2: Request to Transfer Select Agents and Toxins
- APHIS/CDC Form 3: Report of Theft, Loss, or Release of Select Agents or Toxins
- APHIS/CDC Form 4: Report of the Identification of a Select Agent or Toxin
- APHIS/CDC Form 5: Request for Exemption of Select Agents and Toxins for an Investigational Product

APHIS: Animal and Plant Health Inspection Services
CDC: Centers for Disease Control and Prevention
Reproduced from: Centers for Disease Control and Prevention; Animal and Plant Health Inspection Services. Forms. 13 August 2013. http://www.selectagents.gov/Forms.html. Accessed June 25, 2014.

Security Risk Assessment

Security risk assessment (SRA) is the method used to approve an individual for access to select agents or toxins in accordance with the USA PATRIOT Act of 2001 and the Public Health Security and Bioterrorism Preparedness and Response Act of 2002. The Federal Bureau of Investigation Criminal Justice Information Services division determines if an individual who has been identified by a registered entity as having a legitimate need to access a select agent or toxin meets one of the statutory restrictors that would restrict access.

A "restricted person" under 18 USC 175b (USA PATRIOT Act) is an individual[26] who:

- is under indictment for a crime punishable by imprisonment for a term exceeding 1 year or who has been convicted in any court of a crime punishable by imprisonment for a term exceeding 1 year;
- is a fugitive from justice;
- is an unlawful user of any controlled substance;
- is an alien illegally or unlawfully in the United States;
- has been adjudicated as a mental defective or has been committed to any mental institution;
- is an alien (other than an alien lawfully admitted for permanent residence) who is a national of a country as to which the secretary of state has made a determination (that remains in effect) that such country has repeatedly provided support for acts of international terrorism; or
- has been discharged from the armed services of the United States under dishonorable conditions.

All individuals, including the RO, AROs, laboratory research staff, and animal-care workers requesting unescorted access to CDC- or APHIS-registered spaces containing BSAT require an approved SRA. Escorted individuals, such as inspectors and visitors with no access to BSAT, do not require an approved SRA. FSAP works closely with the Federal Bureau of Investigation Criminal Justice Information Services division to identify individuals who are prohibited to access BSAT based on the restrictions identified in the USA PATRIOT Act of 2001.[15] This process involves submitting an amendment to the lead agency (CDC or APHIS) and adding the individual to the entity registration to obtain a unique Department of Justice number, which is recorded on a Bioterrorism Security Risk Assessment Form (FD-961). The completed FD-961 is reviewed, certified by the RO, and submitted to CJIS with two sets of fingerprints.[26] The FSAP authorizes individual access to BSAT based on the results of the SRA. The SRA is renewed every 3 years. All individuals with approved SRA undergo a general initial training, which provides site-specific information on biosafety, security, incident response, and insider threat awareness. Refresher training is provided annually to all SRA-approved individuals.

Biological Select Agents and Toxins Inventory and Accountability

FSAP regulations require complete, current, and accurate inventory of all long-term (LT) BSAT. Materials that contain or have been exposed to infectious select agents, including (but not limited to) laboratory cultures, animals, animal tissues, confirmed clinical specimens, plants, and plant tissues, are subject to FSAP regulations. Select toxins and recombinant or synthetic nucleic acids encoding functional forms of select toxins are also regulated. Animals inoculated with select toxins and their tissues are exempt from FSAP regulations. Inventory records are not required for BSAT that the FSAP has excluded from the provisions of the Select Agent Regulations, nor for inactivated BSAT materials as long as an approved method for inactivation is used.

CDC-DSAT defines LT storage as placement in a system designed to ensure viability for future use. As a rule, LT BSAT materials are not part of an ongoing experiment and have not been accessed for a significant period of time (eg, 30 calendar days).[27] SAT are considered working stock (WS) if the materials are: (*a*) a part of an ongoing experiment, (*b*) accessed frequently, or (*c*) not stored for an extended period of time. FSAP regulations do not require inventory records for BSAT classified as WS; however, all WS must be kept and used in secure locations by approved individuals (ie, those with current SRAs enrolled in a suitability program, if accessing Tier 1 agents). The DA's interim guidance on BSAT inventory management allows BSAT to remain in WS status for up to 180 days; however, the DA guidance document requires individuals to maintain detailed records of all BSAT WS materials at all times.

Significant amounts of BSAT WS can be generated in a containment laboratory on any given day. Accounting for these materials can be challenging, as they are continuously used or consumed in various experiments. Entities with large BSAT inventories must establish procedures to retain only "valuable" BSAT. Establishing peer-reviewed and accepted criteria for retention and destruction of LT BSAT materials can be

beneficial to the investigators and the host entity. An example of criteria developed for retention and destruction of LT BSAT materials is shown in Exhibit 31-3.

Specimen boxes containing LT BSAT materials can be wrapped with tamper-evident materials after verification by two BPRP-certified individuals. Follow-up tube-by-tube inventory is not needed as long as the tamper-evident seals remain intact. Reducing access and repeated contact with LT BSAT materials will preserve specimen integrity and will also allow for accurate real-time inventory of these materials. Additionally, uniform labeling of LT BSAT specimens should remain a priority for research staff in order to have well-labeled research materials for all current and future investigations. Advances in labeling technologies permit for human-readable information, barcodes, and radiofrequency identification tags to be incorporated on any size of specimen labels. Specimen tags that adhere to frozen tubes are also available, making it possible to label archival materials.

Centralized Management of Long-Term Biological Select Agents and Toxins

Maintaining accurate and current inventory of LT BSAT materials at all times can be burdensome to principal investigators (PIs) and research staff who are focused on meeting timelines for deliverables and project goals. BSAT inventory discrepancies identified during internal audits or announced and unannounced inspections by regulatory agencies can result in serious consequences to the registered entity with respect to continuation of research and loss of public trust. One proposed solution to this dilemma is to establish centralized management of LT BSAT materials under the care of the RO and the AROs to alleviate considerable inventory and accountability burden from the PIs and research staff (Exhibit 31-4). Under this model, LT BSAT materials that have been verified by a third party would be labeled with PI-specific information, wrapped with tamper-evident materials, and centrally stored within the registered laboratory space in dedicated storage containers with restricted access.

Centralized LT BSAT inventory management would enhance readiness for unannounced regulatory compliance inspections that include BSAT inventory verification, and would simplify the transition of BSAT inventory when a PI retires or leaves the institution. Verification of LT BSAT inventory by the PI or researcher and a third party would also allow for identification of archival specimens requiring new uniform labels. The PI or researcher will identify BSAT specimens no longer needed for current and future investigations, including potentially contaminated specimens, specimens with reduced or no bioactivity, and excess specimens.

Biological Select Agents and Toxins Inventory Audits

Registered entities are required to conduct complete inventory audits of a PI's BSAT holdings in LT storage during physical relocation of a collection or inventory upon the departure of a registered PI with BSAT holdings, or in the event of a theft or loss of BSAT. In addition to the FSAP requirements, Army Regulation (AR) 50-1 requires annual 100% physical inventory of all BSAT holdings by each PI.[28] If the LT BSAT materials are verified and wrapped, the inventory burden is dramatically reduced, as long as the tamper-evident seals are intact. Army regulation also requires BSAT inventory audits of each registered PI at least once annually by the biological surety program staff. These

EXHIBIT 31-3

SAMPLE CRITERIA FOR RETAINING OR DESTROYING BIOLOGICAL SELECT AGENTS OR TOXINS

Retention Criteria

1. Unique materials (serotypes, strains, etc)
2. Support ongoing research activities and all existing agreements
3. High scientific value for future scientific investigations
4. Deemed evidence material by law enforcement
5. Materials retained from published studies

Destruction Criteria

1. Potentially contaminated and/or degraded materials (eg, samples that have been subjected to multiple freeze/thaw cycles)
2. Excess quantities from a specific microbe or toxin
3. No anticipated future scientific value with the understanding that projected future mission requirements can be difficult
4. Materials that lack expected bioactivity

EXHIBIT 31-4

CENTRALIZED BIOLOGICAL SELECT AGENTS AND TOXINS INVENTORY MANAGEMENT CONSIDERATIONS

Reduce inventory burden on PI/researcher
- Transfer long-term BSAT accountability responsibility to RO/ARO and dedicated biological surety staff (select agent managers)
- Limit principal investigator/researcher responsibility to working stock BSAT materials

Enhance accountability and security
- Manage long-term BSAT materials with dedicated staff
  - 100% long-term BSAT inventory verification and tamper-evident wrapping
  - Long-term BSAT consolidated within registered space
  - Eliminate variability in record keeping from multiple PIs/researchers
- Enhance security of BSAT materials
  - Long-term BSAT in dedicated and locked freezers within registered spaces
  - Limit physical access to long-term BSAT materials

Manageable process with economy of space and personnel
- Enhance real-time inventory awareness for long-term BSAT
- Consolidate long-term BSAT within containment spaces

Maintain mission capability with enhanced flexibility
- Retain all unique and critical BSAT materials
- Capture all essential characterization and experimental data (eg, DoD BSAT database)
- Prepared to receive or send BSAT to other DoD entities at all times

Inventory reduction
- Assist PIs in identifying and destroying BSAT with no current or future scientific value
- Encourage sharing of BSAT among PIs within the institute

ARO: alternate responsible individual; BSAT: biological select agents and toxins; DoD: Department of Defense; PI: principal investigator; RO: responsible official

inventory audits include inspection of laboratory records of BSAT usage, physical inventory verification of both LT storage and WS BSAT, verification of the SAP registration of the PIs, BSAT transfer documentation, and BSAT destruction records. The annual BSAT inventory audits provide a great opportunity to interact with the registered PI and his or her technical staff and to identify areas where additional training may be warranted.

Biological Select Agent and Toxin Transfers

The Select Agent Regulations require entities to develop provisions and policies for shipping, receiving, and storing SAT, including documented procedures for receiving, monitoring, and shipping all SAT. There are primarily two types of BSAT transfers: intraentity and interentity. BSAT material must be packaged by individuals approved by the HHS secretary or APHIS administrator for access to SAT. If the transfer involves Tier 1 BSAT, the approved individuals must be certified in the entity's suitability program or personnel reliability program.

Intraentity transfers of SAT are performed between two registered PIs with a complete chain of custody document. The sender and receiver must be registered with the SAP for the BSAT being transferred. These transfers are physically performed by approved individuals in accordance with entity-specific standard operating procedures. An approved APHIS/CDC Form 2 is not required for intraentity transfer of BSAT materials.

Interentity transfers of SAT require an approved APHIS/CDC Form 2 prior to physical transfer of these materials. Once issued, an approved APHIS/CDC Form 2 is valid for 30 days. These transfers are governed by the US Department of Transportation Hazardous Material Regulations found in 49 CFR, parts 100 to 185.[29] The approved individual packaging SAT must ensure compliance with all applicable laws concerning packaging and shipping. DA uses approved BSP personnel trained and certified in shipping

procedures to verify the contents of the SAT shipments inside the containment laboratories prior to packaging. A completed chain of custody form is retained with copies of shipping documents for at least 5 years (DoD standard). The individual who witnesses packaging inside the containment laboratory also verifies the approved APHIS/CDC Form 2 and the shipping documents. The FSAP has amended the select agent regulations to accept and promote the recommendation of the report of the Defense Science Board Task Force, DoD Biological Safety and Security Program,[30] regarding the "lost in crowd" approach for all SAT shipments. However, registered DoD laboratories are currently required to use a carrier that maintains positive control, ensures chain of custody, is certified to handle HAZMAT (hazardous materials) standards 6.1 (poisons) and 6.2 (infectious substances), and requires two qualified drivers possessing current secret clearance, with at least one driver in the truck or within 25 feet of the truck at all times. Harmonization of DoD regulations with the FSAP is being discussed to standardize the select agent and toxin shipping practices.

Exempt quantity (permissible amount) transfers of select toxins (Table 31-2) are not regulated by the FSAP.[31] The "toxin due diligence" provision was developed by FSAP to address concern that someone might stockpile toxins by receiving multiple orders below the excluded amount. It requires a person transferring toxins in amounts which would otherwise be excluded from the provisions to: (*a*) use due diligence to ensure that the recipient has a legitimate need to handle or use such toxins; and (*b*) report to FSAP if they detect a known or suspected violation of federal law or become aware of suspicious activity related to the toxin.[32]

Most "exempt" toxin transfers are to a nonregistered PI or a collaborator who demonstrates a legitimate need to handle or use the toxin being transferred. Due diligence must precede the transfer to ensure that the recipient does not exceed the exempt quantity limit established by the FSAP with any existing remnant quantities in their laboratories from previous investigations. The person initiating the transfer can require the recipient to complete documentation stating the intended use of the toxins and a statement indicating that receiving the requested amount of the toxin will not put them over the limits established for the select toxins by the FSAP. Tracking "exempt" select toxin transfers (sending and receiving) and monitoring their use must be an integral part of a due diligence effort at the entity level to avoid investigators accumulating quantities of select toxins above the permissible amounts at any time.

TABLE 31-2

PERMISSIBLE TOXIN AMOUNTS

Health and Human Services Toxins	Amount
Abrin	100 mg
Botulinum neurotoxins	0.5 mg
Short, paralytic alpha conotoxins	100 mg
Diacetoxyscirpenol	1,000 mg
Ricin	100 mg
Saxitoxin	100 mg
Staphylococcal enterotoxins (subtypes A, B, C, D, and E)	5 mg
T-2 toxin	1,000 mg
Tetrodotoxin	100 mg

Centers for Disease Control and Prevention; Animal and Plant Health Inspection Services. Permissible Toxin Amounts. 5 October 2012. http://www.selectagents.gov/Permissible%20Toxin%20Amounts.html. Accessed June 25, 2014.

Reporting Theft, Loss, or Release of Biological Select Agents and Toxins

FSAP requires an entity to immediately notify CDC or APHIS and appropriate federal, state, or local law enforcement agencies (by e-mail, facsimile, or telephone) of incidents involving theft, loss, or release (occupational exposure or release of an agent or toxin outside of the primary barriers of the bio-containment area) of SAT.[33] Thefts or losses also must be reported even if the SAT is subsequently recovered or the responsible parties are identified. A completed APHIS/CDC Form 3 must be submitted within 7 calendar days.

A BSAT inventory deficiency investigation may involve: (*a*) immediate notification to the physical security office; (*b*) 100% physical inventory of all of the registered PI's BSAT holdings by the RO or ARO; (*c*) complete inspection of the PI's BSAT usage records (laboratory notes); and (*d*) a complete database records check of the BSAT inventory holdings of the PI. If theft of BSAT is suspected, appropriate law enforcement agencies must be informed.

Release of BSAT from "primary containment" or release resulting in "potential exposure" to individuals requires immediate notification to the FSAP. Spills of SAT in biological safety level (BSL)-4 laboratories (sealed laboratories with personnel wearing positive pressure encapsulated suits) can be safely cleaned up without potential human exposure; no FSAP reporting is necessary because the entire BSL-4 laboratory

is considered "primary containment." However, if an individual experiences a breach in his or her positive pressure encapsulating suit at the same time as a spill or work done with animals outside of primary containment, initial notification to FSAP reporting is required, followed by the completion of APHIS/CDC Form 3. In contrast, SAT spills in BSL-2 and BSL-3 laboratories (unsealed directional airflow laboratories with personnel not wearing positive pressure encapsulated suits) outside of a functioning biological safety cabinet are reportable to FSAP, as these laboratory spaces are considered "secondary containment." The data collected and analyzed by the CDC on theft, loss, or release reporting from 2004 to 2010 indicate that the risk of exposure from BSAT managed by US laboratories to the general population is low.[34]

Identifying Select Agents and Toxins

Identifying BSAT as a result of diagnosis, verification, and proficiency testing, and final disposition of the identified agent or toxin must be reported to FSAP within 7 calendar days by completing APHIS/CDC Form 4. Identifying Tier 1 BSAT (see Exhibit 31-2) from diagnostic samples requires immediate (ie, within 24 hours) reporting to FSAP via e-mail, facsimile, or telephone. BSAT identified from proficiency testing specimens must be reported within 90 days of receipt of the sample. Any amount of select toxin identified must be reported to FSAP. Entities not registered with the FSAP are also required to report BSAT that have been identified from diagnostic specimens. Unregistered entities have 7 calendar days to transfer to a registered entity or destroy the identified SAT to remain in compliance with current federal regulations.

Restricted Experiments

An individual or an entity approved by the FSAP may not conduct restricted experiments without prior approval by the HHS secretary or APHIS administrator. Restricted experiments are: (*a*) experiments that involve the deliberate transfer of, or selection for, a drug resistance trait to select agents that are not known to acquire the trait naturally, if such acquisition could compromise the control of disease agents in humans, veterinary medicine, or agriculture; and (*b*) experiments involving the deliberate formation of synthetic or recombinant nucleic acids containing genes for the biosynthesis of select toxin lethal for vertebrates at an LD_{50} (the amount necessary to kill 50% of the subject population) that is less than 100 ng/kg body weight.[19] Additional guidance on restricted experiments involving recombinant or synthetic nucleic acids is outlined in the NIH's *Guidelines for Research Involving Recombinant or Synthetic Nucleic Acid Molecules*.[35] This guidance is mandated for research that is conducted at or sponsored by an entity that receives any support for recombinant or synthetic nucleic acid research from the NIH, including research performed directly by the NIH.

Most registered entities designate the responsibility of identifying, reviewing, and approving restricted experiments to their Institutional Biosafety Committee (IBC). The biosafety officer and the RO are members of the IBC. Entity-specific IBC-approved research proposals with restricted experiments are forwarded to FSAP for review and approval. Restricted experiments containing HHS and overlap select agents will be further reviewed by the Intragovernmental Select Agents and Toxins Technical Advisory Committee. Restricted experiments involving USDA select agents will be further reviewed by subject matter experts from APHIS.

A typical request to FSAP to review a restricted experiment includes, but is not limited to, description of:

- the proposed experiment, including intended objectives,
- nucleic acid insert and the intended biological characteristics of the recombinant gene product,
- cloning/expression vector,
- host organism used for molecular cloning,
- selection methods (recombinant or passive),
- antimicrobial markers use,
- BSL considerations,
- estimated amount of toxin (recombinant or synthetic) to be produced (if applicable), and
- any planned animal or plant experiments.[36]

Restricted experiments using recombinant and passive selection methods and all select agent products resulting from these experiments are also subject to FSAP regulations. Transfer of any products of restricted experiments must be coordinated through the FSAP. The DA and the DoD require all of their research laboratories to remain in full compliance with all federal regulations governing BSAT.

Biosafety

Biosafety in microbiological and biomedical laboratories is based on two key principles: "containment" and "risk assessment." Core concepts of containment include microbiological practices, safety equipment, and facility safeguards that protect laboratory workers, the environment, and the public from exposure to infectious organisms. Risk assessment is a process that enables the appropriate selection of microbiological

practices, safety equipment, and facility safeguards that can prevent laboratory-associated infections. Modern biosafety practices described in the 5th edition of *Biosafety in Microbiological and Biomedical Laboratories*[37] are accepted as standards of practice by all CDC-registered entities to conduct work with SAT. The DA mandates the use of the current version of the manual and DA pamphlet 385-69, *Safety Standards for Microbiological and Biomedical Laboratories*,[38] in all US Army activities and facilities in which infectious agents or toxins are used, produced, stored, handled, transported, transferred, or disposed of, including the Army National Guard, the US Army Reserve, and contractors and consultants conducting microbiological and biomedical activities for the Army. The detailed principles and practices of biosafety are covered in a separate chapter of this textbook.

It is critically important to thoroughly train individuals in biosafety practices prior to providing access to the containment laboratories to handle, manipulate, and store BSAT. Training must include:

- microbiological laboratory techniques;
- use of personal protective equipment (PPE), safety equipment, and containment laboratory equipment;
- information on bloodborne pathogens;
- an entity-specific chemical hygiene plan;
- BSAT-specific information;
- emergency exit operations;
- immediate first aid; and
- reporting requirements for potential exposures to infectious agents and toxins.

Some of this initial training can be structured into mentorship programs in which individuals approved as mentors ensure new laboratory workers are able to work safely within the containment laboratories before they are granted independent access. In addition to project-specific training, the worker requesting access to the containment laboratories with BSAT is trained to recognize biohazards, understand potential health risks associated with exposures, provide appropriate first aid, and carry out follow-up reporting procedures.

FSAP regulations require individuals with access to Tier 1 BSAT to be enrolled in an occupational health program.[19–21] AR 50-1[28] also requires commanders and directors of the entities with a biological surety mission to establish and implement an occupational health program. Core elements of an occupational health program include: (*a*) risk assessment, (*b*) medical surveillance, (*c*) access to clinical occupational health services and management, and (*d*) hazard communication. Select agent risk assessments should consider:

- route of exposure,
- infectious dose,
- agent virulence,
- incubation period,
- environmental stability,
- communicability,
- genetic modification,
- available resources for pre- and post-exposure prophylaxis,
- available vaccine options,
- PPE use, and
- biocontainment requirements.[39]

Occupational health plans are required to comply with US Department of Labor and Occupational Safety and Health Administration regulations, as well as patient confidentiality laws. Promoting a safe and healthy work environment requires limiting exposures to infectious agents and toxins, promptly detecting and treating exposures, and using information gained from incidents to further improve safety measures and worker training. Occupational health and safety is a shared responsibility among the individual workers, their supervisors, PIs, biosafety specialists, healthcare providers, and the employer.

Personnel Reliability

Personnel reliability programs existed for decades in the US military. The BPRP, modeled after the military's nuclear and chemical personnel reliability programs, ensures that individuals with access to BSAT meet the highest standards of reliability.[18,40–42] The concept of personnel reliability was implemented over a decade ago in DoD laboratories working with BSAT.[43] AR 50-1, *Biological Surety*, outlines the BPRP described herein.[28]

Individuals with access to BSAT in DA and DoD laboratories are required to be enrolled in a BPRP. The FSAP added the "suitability" requirement for individuals with access to Tier 1 BSAT in October 2012. The FSAP's suitability assessment of personnel is based in part on the DoD's personnel reliability programs. The BPRP and the suitability assessment of personnel are primarily designed to reduce the risk of SAT misuse by an individual who has access to these agents (insider threat). The intent of the US Army's BPRP[18] and the FSAP's suitability assessment of personnel[44] is the same; however, there are significant differences between the two programs.

The commander or director is the head of the organization's BPRP and can serve as the reviewing official. The reviewing official appoints certifying officials (COs) who determine the reliability and suitability of individuals requiring access to SAT and ensure they

are appropriately qualified and trained to perform their duties. Commanders and directors may appoint BPRP monitors to assist COs in administering day-to-day activities; however, COs are responsible for continuous monitoring of individuals enrolled in the personnel reliability program. The reviewing official monitors the CO's decisions to disqualify individuals and may overturn them when procedures are unfair, inconsistent, or incorrectly applied. AR 50-1 requires the reviewing official to review all individual disqualification actions submitted by the CO.[28] The FSAP recommends suitability decisions on individuals requesting access to Tier 1 BSAT be a combined decision of the CO, RO, and the entity leadership (eg, commander, director, or reviewing official).[44]

To begin enrollment in the BPRP, supervisors of individuals who need to access BSAT in the CDC-registered containment spaces contact the designated CO. The CO is the gatekeeper for access to BSAT, ensuring that persons requesting access have met all the qualifying conditions. FSAP ensures that restricted persons do not have access to BSAT through the SRA process (see section above); the Army's BPRP further ensures that persons with access to BSAT are:

- trustworthy,
- mentally and emotionally stable,
- physically competent,
- free of unstable medical conditions,
- able to exercise sound judgment,
- willing to accept responsibility,
- able to adapt to changing work environments,
- free from drug and alcohol abuse,
- willing to participate in random drug testing, and
- willing to comply with all training requirements.

Enrollment in the Army's BPRP involves:

- initial interview,
- personnel records review,
- personnel security investigation,
- medical evaluation,
- drug testing, and
- CO's final evaluation and briefing.

The order of steps in the process is discretionary; nevertheless, each step must take place and be fully documented.

Initial Interview

The CO is required to conduct a personal interview of a potential enrollee in the BPRP to assess suitability and reliability. The CO must inform the candidate of the Privacy Act of 1974[45] and the Health Insurance Portability and Accountability Act[46] to obtain consent to proceed with the screening process. Although not required by the regulations, the initial interview may also include a written questionnaire. The candidate is asked questions that will allow the CO to determine whether he or she has engaged in any activities that would be either mandatory or potentially disqualifying factors. Mandatory disqualifying factors are those that are beyond the discretion of the CO for deciding reliability and suitability. If extenuating circumstances exist, the reviewing official may request an exception for the individual's enrollment through command channels. The following are mandatory disqualifying factors:

- current substance or alcohol dependence;
- drug or substance abuse within 5 years prior to the initial interview;
- trafficking, cultivating, processing, or manufacturing illegal or controlled drugs within the past 15 years;
- drug or substance abuse while enrolled in BPRP;
- inability to meet safety requirements; or
- meeting the criteria of a restricted person as defined by 18 USC § 175b.[47]

Other potentially disqualifying factors include:

- alcohol-related incidents or alcohol abuse;
- drug or substance abuse more than 5 years prior to initial interview; or
- mental or physical medical condition, medication usage, or medical treatment that may result in:
  - altered state of consciousness,
  - impaired judgment or concentration,
  - increased risk of impairment if exposed to BSAT,
  - impaired ability to wear PPE,
  - inability to meet physical requirements, or
  - inappropriate attitude, conduct, or behavior.

The CO must inform the candidate that he or she will be subject to random, unannounced drug testing as part of continuous monitoring; an initial negative test is required prior to certification in the BPRP. The CO must also explain to the candidate about: (*a*) continuous monitoring, (*b*) the requirement for self-reporting, and (*c*) use of prescription drugs. The initial interview is a good opportunity for the CO to get to know the candidate and to begin a relationship based on mutual trust and respect.

Personnel Records Screening

Once the CO has completed the initial interview and found the candidate to be suitable for enrollment, the applicant's personnel records are screened by a supporting personnel officer. The screening official will determine the individual's citizenship and identify it to the CO. Any potentially disqualifying information (PDI) discovered during the screening process is immediately communicated to the CO. Individuals with extended federal government service may have information in their personnel records from the inception of their employment. In contrast, information in contract employees' personnel records may be limited to the length of their employment with that company. Anything that may indicate unsatisfactory employment history or dereliction of duty, such as job applications, enlistment contracts, and any other available pertinent record should be reviewed for PDI. The CO acts on any PDI discovered during the personnel records screening; however, the CO does not retain any records of this information.

Personnel Security Records Screening

The current minimum personnel security investigation (PSI) requirement for unescorted access to BSAT within DoD is a favorably adjudicated single-scope background investigation. This level of PSI is conducted to confer top secret clearance. However, a security clearance is not required for BPRP enrollment. The personnel security manager will request a copy of the PSI from the Office of Personnel Management on behalf of the CO. The personnel security officer will expeditiously provide any adverse information to the CO, ensuring Privacy Act requirements are not violated. Personnel scheduled for initial assignment to BPRP positions must have the appropriate and favorably adjudicated PSI completed within the 5 years preceding certification to the BPRP. PSI files contain sensitive information and should only be retained for the time necessary to determine suitability and reliability. The CO will review the results of the personnel security investigation to determine if the individual meets the suitability and reliability requirements of the BPRP.[48] The FSAP is not prescriptive, with respect to PSI, above what is required to obtain an SRA for suitability assessment of individuals with access to Tier 1 BSAT.

Medical Evaluation

The competent medical authority (CMA) medically evaluates the candidate to ensure that the individual seeking enrollment in the BPRP is physically, mentally, and emotionally stable; alert; competent; dependable; and free of unstable medical conditions that may impact BPRP duties.[18] The CMA meets with the candidate and reviews the individual's medical records to identify any PDI. Medical PDI includes any medical condition, medication use, or medical treatment that may result in an altered level of consciousness, impaired judgment or concentration, impaired ability to safely wear required PPE, or impaired ability to perform the physical requirements of the BPRP position, as substantiated by the medical authority to the CO. The candidate may also provide the CMA copies of medical records from a personal healthcare provider. If medical records are incomplete or inadequate, the CMA will conduct the appropriate medical evaluation. This may include a mental health evaluation if the CMA determines such an evaluation is prudent or upon request by the CO.[18] Medical PDI is reported to the CO with recommendations regarding the person's fitness for assignment to these duties or limitations in duties or reasonable accommodations that might allow the individual to perform his or her duties without compromising worker safety.

Drug Testing

All candidates for BPRP must complete drug testing within 6 months prior to initial certification. All drug test results will be provided to the CO before the individual is certified in the BPRP. Positive drug test results indicating illegal drug use will result in disqualification.

Certifying Official's Final Evaluation and Briefing

After the candidate has completed all phases of the screening, the CO conducts a final evaluation of all the information received during the screening process and conducts a final interview. During the final interview, the candidate will have an opportunity to review and discuss any BPRP-relevant issues, including PDI discovered during the screening process and the circumstances surrounding such an event, and before the CO's decision on the candidate's suitability and reliability for the program. During this time the CO:

- reviews the duties and responsibilities of the individual's BPRP position, including required PPE use;
- discusses the expectations for continuous monitoring;
- reviews disqualifying factors, including any incidents or medical issues that may have occurred since the initial interview;

- reminds the individual that prescription drug use must be under the supervision of a health-care provider; and
- reviews the self-reporting requirements of the BPRP.

At the end of the interview, the CO should inform the candidate whether he or she is suitable for the program, and the individual signs DA Form 3180 indicating his or her understanding of the program and willingness to comply with the requirements. If the candidate is determined to be eligible, the CO ensures that the candidate has completed all the core safety, security, and emergency training. The CO will notify the RO immediately after the individual is certified in the BPRP.[18]

Individuals certified in the BPRP are subject to continuous monitoring. Continuous evaluation includes, but is not limited to:

- self-reporting,
- peer and supervisor observation and reporting,
- periodic unannounced drug testing,
- periodic personnel security investigations,
- periodic medical evaluations by the CMA, and
- CO observation and evaluation.

The FSAP recommends the RO's involvement in the development, implementation, and administration of the Tier 1 BSAT suitability assessment program. The RO must ensure that access to Tier 1 BSAT is limited to individuals in the suitability program with the entity's ongoing suitability monitoring, and have current FSAP approval to access SAT. Ongoing efforts to harmonize the DoD regulations governing BSAT with the FSAP are expected to clarify the role of the RO in the BPRP or suitability assessment program.

Biosecurity

Safeguarding BSAT is a high priority for the DoD[41,43] and the FSAP.[49] According to the Office of Science and Technology Policy, the term "biosecurity" refers to the protection, control of, and accountability for high-consequence biological agents and toxins and critically relevant biological materials and information within laboratories to prevent unauthorized possession, loss, theft, misuse, diversion, or intentional release.[50] AR 190-17, *Biological Select Agents and Toxins Security Program*[51] prescribes the policy, responsibilities, procedures, and minimum standards for safeguarding BSAT. Biosecurity plans are based on risk assessments, are entity specific, and constitute sensitive information. A site-specific security plan based on risk assessments must be developed by all CDC-registered entities with BSAT. An effective site-specific security plan will have initial and continuous input from and interactions with:

- security personnel,
- commanders or directors,
- subject matter experts,
- local law enforcement officers,
- ROs and AROs,
- biosafety officers,
- occupational health CMAs,
- facilities management personnel, and
- information security management personnel.

An effective biosecurity plan is based on operational processes, accounts for all BSAT from creation or acquisition to destruction, does not violate any laws, weighs both primary and secondary affects, and is reviewed and updated at least annually.

The biosecurity program for CDC-registered entities with BSAT can be broadly divided into at least five major components: (1) BSAT security, (2) physical security, (3) personnel security, (4) operational security, and (5) information security.

Biological Select Agents and Toxins Security

There are a number of factors that contribute to the challenge of effective BSAT inventory and accountability within containment laboratories. Temperature-sensitive microbes, confined spaces, sharing of limited freezer space by multiple investigators, co-existence of both LT and WS BSAT, multiple users, and illegible specimen labels can all contribute to ineffective BSAT inventory and accountability. Uniform labels with human-readable information and barcodes, inventory verification and wrapping of all LT BSAT with tamper-evident materials, centralized storage of wrapped LT BSAT within the containment laboratory, and controlled access to LT BSAT materials can preserve the integrity of the stored specimens and provide an accurate real-time inventory of these materials. These LT BSAT management strategies can be instituted without affecting ongoing research. Entities must establish standard operating procedures for incoming, outgoing, and intraentity BSAT transfer. All transfers must be conducted with chain-of-custody documentation, which is retained and verified with BSAT inventory databases. BSAT destruction documents should be confirmed with the BSAT databases. BSAT inventory audit should include review of laboratory notes and verification of BSAT WS materials. All BSAT materials

must be maintained in CDC-registered laboratory spaces with restricted access to prevent theft, loss, or release of these materials. All personnel with access to BSAT must be trained in FSAP regulations, including reporting requirements. Entities must also conduct a complete inventory audit of a PI: (*a*) when the PI with BSAT holdings leaves the entity; (*b*) in the event of a theft or loss of BSAT; and (*c*) upon physical relocation of a collection of BSAT materials. These practices will also prepare the entity for any unannounced inspections. Effective BSAT inventory and accountability practices will preserve the integrity of the specimens and increase research efficiency within the containment laboratories.

Physical Security

A physical security plan developed using site-specific risk assessment can detect, deter, or delay threat and provide sufficient time to respond to the threat. Security barriers such as perimeter fences, armed guards, walls, locked doors, secured laboratories, and locked freezers can deter intrusion and deny access to BSAT. FSAP regulations require:

- controls limiting access to CDC-registered spaces to approved individuals with access to BSAT,
- provisions to safeguard animals and plants infected with select agents,
- review and update of access logs to CDC-registered spaces,
- prevention of access credentials sharing,
- procedures for reporting loss of access credentials,
- procedures for personnel changes,
- three barriers (physical structures that are designed to prevent access to unauthorized individuals) to access Tier 1 BSAT,
- intrusion detection systems where Tier 1 BSAT is manipulated or stored,
- response time not exceeding 15 minutes for a force capable of interrupting a threat to Tier 1 BSAT manipulation and storage spaces, and
- procedures for access control in power failures.[19,49]

Personnel Security

The FSAP and DoD consider personnel security integral to detecting insider threat. The personnel security office at the entity level works with the RO to facilitate SRA documentation and fingerprinting for individuals requesting access to CDC-registered spaces. Personnel security also includes: verification of background information, security investigations, personnel dossier reviews, identifying violators of security and safety procedures, and identifying individuals who threaten or support those who threaten to do harm to others. The biosecurity plan should include personnel security measures based on a site-specific risk assessment. A robust "insider threat awareness" training program developed and continuously updated based on site-specific risk assessments is administered to individuals with access to Tier 1 BSAT. Insider threat awareness training is an annual requirement.[19]

Operational Security

Effective operational security posture builds on existing operational procedures and mitigates threats based on site-specific risk assessments.[49] Operational security measures for an entity with BSAT should include:

- training personnel on securing BSAT;
- monitoring individual access to areas containing SAT;
- monitoring BSAT activities inside containment suites through security closed-circuit television or by using an escort;
- control of after-hour and weekend access to containment laboratories with BSAT;
- screening visitors, packages, and delivery trucks at the entry point;
- procedures in place for immediate notification to the RO, commander or director, security forces, and law enforcement if theft or loss of SAT is suspected;
- training personnel to identify and report suspicious activities;
- prominently displayed identification badges on individuals within the entity;
- constant building security surveillance;
- intrusion detection systems;
- surveillance of backup power generators; and
- peer reporting procedures for any sudden changes in behavior among approved individuals with access to SAT.[49]

Information Security

FSAP regulations require registered entities to develop and implement procedures for information control and information security.[52] Information security procedures and protocols must:

- ensure all external connections to systems that manage security for the registered space are isolated or have controls that permit and monitor authorized and authenticated users;
- ensure authorized and authenticated users only access information necessary to fulfill their roles and responsibilities;
- prevent malicious code from compromising the confidentiality, integrity, or availability of information systems that control safety and emergency equipment, engineering controls for the containment laboratories, and access to registered space;
- include regular patching and updates to operating systems as well as to individual applications;
- protect network operating systems with security firewalls;
- protect hardware assets;
- include data encryption;
- ensure remote access capability;
- establish robust information backup systems in the event of primary system failure;
- establish procedures for purging electronic storage media prior to disposal; and
- establish procedures for shredding paper documents and computer disks.[52]

Incident Response and Emergency Management

A robust incident response plan and a knowledgeable and competent emergency management team are critical to an entity involved in developing medical countermeasures against dangerous pathogens and toxins. An incident is an occurrence, natural or human-made, that requires a response to prevent the theft, loss, or release of an SAT or to protect human life and animal and plant health.[53] FSAP, DA, and DoD regulations require entities with SAT to develop, exercise, and routinely update a comprehensive, site-specific incident response plan to ensure the security and safeguarding of BSAT in the event of human-made threats and natural disasters. A site-specific incident response plan protects human life before property, is focused on laboratories and not just the entire facility, is developed as a result of collaboration between research staff and leadership, includes responder participation and training, and addresses primary and secondary effects and the impact on workers at the facility.[53] Developing an incident response plan at the entity level should be a team effort involving (but not limited to) the RO, AROs, biosafety officer, facility engineers, PI or researcher, security manager, occupational health physician or CMA, and entity leadership, with input from local first responders (fire department, emergency medical and law enforcement).

Laboratory leadership, supervisors, biosafety specialists and subject matter experts within a registered entity with SAT should develop incident response information specific to the agents, toxins, and procedures conducted in that laboratory. Individuals working in the laboratory must be trained on how to respond to an incident with the materials they handle in the laboratory, emergency exit procedures, and the use of communication devices within the laboratory. Laboratory incident response information must also include decontamination protocols, first-aid, and reporting requirements.[19] Laboratory and facility incident response plans should be practiced via exercises with entity staff and external first responders (fire department, emergency medical and law enforcement); this practice is critical and will save lives and property in the event of a real incident.

The incident response plan should consider and mitigate vulnerability assessments specific to the laboratory and the facility. The incident response plan must include provisions for theft, loss, or release of SAT, inventory discrepancies, and security breaches.[19]

Theft, Loss or Release

Response to suspected theft or loss of SAT should include immediate notification to the entity RO and commander or director for an immediate investigation and verification of pertinent SAT inventory. An investigation should include physical inventory and reconciliation of all LT SAT with database records, review of laboratory usage records, transfer records, destruction records, and WS records. Once theft or loss has occurred, the investigation and recovery of SAT is a law enforcement function. Law enforcement, state, and federal agencies, including FSAP, must be notified of theft or loss of SAT; in terms of FSAP, initial notification is followed by a completed APHIS/CDC Form 3 within 7 days. The entity should be prepared to support law enforcement with all its recovery efforts.

Release of SAT from primary containment could occur during movement (breakage of specimen tubes), due to loss of engineering controls (eg, equipment malfunction, power outage), or as a result of an unforeseen event inside the containment laboratory. SAT release can pose a significant additional risk of exposure to workers if they are not adequately protected with PPE and if the release is not captured and neutralized. Workers potentially exposed to SAT should be immediately evaluated by occupational medicine staff, and appropriate follow-up care must be provided to the affected workers.

Local and state public health agencies and FSAP must be notified of SAT release, including potential exposures to workers. Theft, loss, or release of SAT is also reported to the chain of command in the DA and DoD laboratories.

Inventory Discrepancies

SAT inventory discrepancies (overage or shortage) should be immediately reported to the entity RO and AROs. The PI and research staff must conduct an investigation to resolve or confirm the inventory discrepancy. The memorandum of inventory discrepancy investigation should include:

- identity of the SAT,
- amount of discrepancy,
- date of last inventory and by whom,
- current or last known storage location,
- names of individuals who discovered the discrepancy,
- names of individuals who are notified of the discrepancy, and
- explanation or resolution, if available.

Theft and loss of BSAT must be reported to FSAP.[19]

Security Breaches

A security breach can occur due to a disruption in an established security network or failure to follow established security procedures and policies, or during active and deliberate intrusion from unauthorized sources (eg, intruders, enemy forces). The RO and the commander or director must be notified of all security breaches to restricted areas containing SAT. Security breaches may include:

- access to SAT by individuals not approved by the FSAP;
- individuals "piggy backing" into restricted areas;
- tampering of access controls, locks, and seals securing SAT;
- unauthorized access to SAT inventory databases;
- tampering of security badges, passcodes, or other entry credentials to restricted areas containing SAT;
- unauthorized removal of SAT from restricted areas;
- sharing of access credentials by workers;
- damage to building infrastructure resulting in easy access to SAT; and
- compromises due to hacking or deliberate manipulations in computer programing controlling containment access.

Lessons learned should be incorporated to enhance security systems and decrease security breaches.[56] The FSAP requires the RO to ensure that individuals with access to SAT are trained annually on entity incident response plans.

SUMMARY

The intent of the FSAP and the DoD's BSP is the same: to allow peaceful research to continue while restricting BSAT access to individuals and parties who intend to misuse them and do harm. Overall, current regulatory requirements promote laboratory safety and security of BSAT by requiring laboratory registration; prescreening of individuals requesting access to BSAT; personnel reliability or suitability assessments for individuals seeking access to Tier 1 BSAT; BSAT inventory management; preapproval and monitoring of BSAT transfers; reporting requirements for theft, loss, release, or identification of BSAT; preapproval for certain genetic alterations to BSAT (restricted experiments); and periodic onsite inspections by regulatory agencies. Regulatory burden on entities with BSAT can be significant; however, it is critical for the public to have confidence that work involving BSAT is conducted in a manner that prioritizes laboratory and public safety and protection of the environment.

Biological surety and security requirements to access BSAT in DoD laboratories currently meet or exceed that of the FSAP. DoD also imposes additional biological surety and security measures beyond those required by the FSAP, on contractors using DoD-owned BSAT. Having different eligibility standards to access and work with BSAT can have significant impact on collaborative research; harmonization of administrative policies and practices of facilities registered with FSAP is expected to promote increased collaboration among scientists. Currently, DoD is synchronizing its biological surety regulations with the FSAP regulations in accordance with Executive Order 13546.[22]

Scientific advances in synthetic biology are likely to challenge the current regulations governing BSAT; however, current US regulations governing BSAT are consistent with the broad international framework of agreements intended to prevent development and proliferation of chemical and biological weapons.

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Chapter 32

ETHICAL ISSUES IN THE DEVELOPMENT OF DRUGS AND VACCINES FOR BIODEFENSE

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DILEMMAS FOR BIODEFENSE RESEARCH

SUMMARY

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INTRODUCTION

The anthrax postal mailings of October 2001 made the nation acutely aware of not only the possibility of biological weapons attacks on US soil, but also brought to the forefront concerns over the proper measures to be implemented to prepare for and prevent such biological warfare scenarios. It is evident that drugs and vaccines may be needed immediately to respond appropriately to emergency or battle situations; however, definitively identifying a realistic threat list and implementing a coherent, organized strategy of biodefense remain elusive. Government research funding and regulatory agencies, intelligence gathering agencies, private and government-sponsored pharmaceutical industries, and the armed services must work together more effectively to accurately identify the immediate and future threats so that countermeasure therapeutics—and in a limited fashion vaccines—are prioritized for research, development, and production. Many potential drugs that are not yet approved for marketing but have preclinical evidence of efficacy may be considered and used in the event of bioterrorist attacks or in times of war.

The pharmaceutical industry is not prepared or organized to respond to such situations; it is in the business of developing drugs to treat natural diseases afflicting patients of the civilian healthcare industry. Profit considerations and sustained business growth are the primary objectives of pharmaceutical companies and their shareholders, so drugs are more likely to be developed for common and chronic diseases rather than rare diseases. For such naturally occurring, often relatively common diseases, many potential test subjects are available to participate in drug safety and efficacy trials because of the possibility that the new drug might cure their diseases or help future patients.

This is not the case for products required as countermeasures against biological warfare agents. These infectious disease agents and toxins are usually found in areas of the world where they occur in sporadic, small epidemics that kill everyone affected and fail to spread. In any case, there are rarely sufficient numbers of "naturally" occurring disease outbreaks of this kind to conduct clinical trials yielding substantial evidence of human clinical efficacy. To fulfill this critical need to generate efficacy data on the product of interest, the US Food and Drug Administration (FDA) established the animal efficacy rule under 21 CFR [Code of Federal Regulations] 314.6. This regulation allowed the FDA to approve the use of an investigational drug in humans based on evidence of effectiveness from studies conducted using well-controlled, validated animal models.[1,2]

Over the past 60 years the conditions that must be met to use many of these drugs and vaccine products have become more restrictive. Until the approval of an animal efficacy rule and passage of the Project BioShield Act of 2004, FDA regulations originating in the 1938 Food, Drug, and Cosmetic Act made emergent medical responses to bioterrorist attacks extremely complex by prohibiting use of investigational products until there was substantial evidence of human clinical efficacy. Gathering evidence in a scientifically valid clinical trial requires the participation of large numbers of subjects who have or are at risk of acquiring the disease, and accumulating these clinical observations requires a lot of time and considerable expense. Although some disease agents cause sporadic epidemics, others infect individuals randomly when a reservoir of contagion is present. Biowarfare attacks involving these highly infectious agents would likely affect many people suddenly, permitting neither the opportunity to enroll enough subjects in a study nor the time to establish the needed medical resources for detailed clinical observations.

Although FDA restrictions are meant to protect the public from possible harm, delaying use of potentially beneficial products until clinical efficacy trial outcomes are known can be detrimental to a prompt and effective response in the event of a widespread biowarfare attack. Throughout most of the 20th century and into the 21st century, successful animal studies followed by substantial evidence of efficacy from human clinical trials have been required before a drug could be approved for market. In an emergency, however, it may be beneficial to allow animal study evidence if the circumstances cannot permit controlled human drug efficacy trials.

Current regulations governing research related to biodefense development cover a wide swath of legal and ethical ground. However, the relationship between the military and the FDA is a complex one, partly because of the institutions' different missions. The FDA regulates the manufacture, testing, promotion, and commerce of medical products, and it makes a legal distinction between products that are approved and not approved for marketing. Products with FDA approval for testing but not approved for marketing are classified as investigational new drugs (INDs). FDA regulations specify what is necessary to move from an investigational product to an approved drug. Under directive Department of Defense (DoD) Instruction 6200.02, dated February 27, 2008, the DoD shall "make preferential use of products approved by the FDA for general commercial

marketing, when available, to provide the needed medical countermeasure;" however "if at the time of the need under a force health protection program for a medical countermeasure against a particular threat, no satisfactory FDA-approved medical product is available, request approval by the ASD(HA) [Assistant Secretary of Defense for Health Affairs] to use an unapproved product under an EUA [emergency use authorization] or, if an EUA is not feasible, under an IND [investigational new drug] application."[3]

Because members of the armed services are at the greatest risk for biowarfare attack, it is prudent for the military to research and develop effective biological defenses that may also be used for treatment in the civilian population in an emergency. FDA regulations pose three significant legal hurdles to the military's ethical responsibility to protect service personnel. First, because organisms that are potential biological warfare agents, such as those causing inhalational anthrax, plague, or Ebola, are rare in nature (generally producing small, sporadic clusters of disease with case numbers insufficient for field trial efficacy studies) and can be life threatening, it is immoral to conduct clinical trials to determine clinical efficacy because of the inherent risk to participants. Second, outside of clinical trials, the systematic use of IND products (as opposed to single use instances) in emergency life-threatening situations is illegal. Third, it is illegal for the DoD to systematically use licensed drugs in large numbers of persons for uses other than those granted by the FDA product licensure agreement (off-label use) without the notice required under 10 USC [United States Code] 1107(1) or under the legitimate practice of medicine. Ultimately, however, researchers must find ways to overcome these limitations so that the FDA and DoD can fulfill their respective executive branch responsibilities while minimizing conflicts.

Federal regulations serve as practical and praiseworthy legal and ethical safeguards for the conduct of human subjects' research. However, as detailed above, regulations governing the conduct of human subjects research can also have the unintended consequence of slowing the development and advancement of biodefense-related medical products, which can result in the following ethical dilemma: on one hand, the military has the duty to adhere to regulations and obey the country's laws; on the other hand, the military has the duty to use all available means to protect its personnel and civilians and accomplish the mission. Mechanisms to bridge the two horns of this dilemma are needed; in particular, there must be a legal way to make protective drugs and vaccines available when the normally required clinical trials cannot be conducted. Consequently, several avenues have been established to address these issues including the use of notice under 10 USC 1107(a), the use of the EUA provisions of section 564 of the Federal Food, Drug, and Cosmetic Act, enacted in 2004 by the Secretary of Health and Human Services, or the establishment of an FDA approved IND protocol.[3,4]

This chapter will demonstrate ways to protect military personnel and possibly even the civilian population. The history of the development of biodefense in military medicine and the ethics of biomedical research will be covered. In addition, a summary of the evolution of regulations that influence or inform human subjects research, including research intended and designed in part to meet the needs of the military personnel, will be presented. Then an analysis and discussion of the conflict between regulatory requirements and adherence to ethical principles in the military setting will demonstrate three options the DoD may pursue in relation to the issues outlined. Some of the legislated solutions recently proposed or implemented will also be included.

OVERVIEW OF THE HISTORY OF BIODEFENSE DEVELOPMENT AND MEDICAL ETHICS

Advances in biomedical research have led to considerable breakthroughs in the treatment of diseases that military personnel face. Although the focus of this chapter is on biodefense, the history of research to protect military personnel from disease has frequently targeted naturally occurring diseases unfamiliar to US troops. The need for development of medical treatment in military settings has frequently been the impetus for conceptual breakthroughs in the ethics of human participation in research. Biomedical research involving human subjects in military research facilities must be conducted with oversight from an institutional review board (IRB), per 32 CFR 219.109,[5] 45 CFR Part 46,[6] 21 CFR 56,[7] and 21 CFR 50. Acknowledgment of ethical dimensions in biodefense research requires the cooperation of all military personnel. However, the ethical principles that serve as the foundations of current ethical practices in military medical research did not come about de novo, and neither did the biodefense and protection methods. Military medical ethics standards evolved over centuries, often in tandem with or in reaction to biodefense needs, or in response to ethical lapses or controversies. At times the military has assumed the lead in establishing human subjects' research ethics precedence.

Biodefense and Ethics in the 18th and 19th Centuries

In 1766, while still a general for England, George Washington and his soldiers were unable to take Quebec in the French and Indian War. In part this failure was due to smallpox outbreaks that affected his troops.[8] Later when Washington led Continental Army troops against the British, a smallpox epidemic reduced his healthy troop strength to half while the British troops, who had been variolated, were already immune to the spreading contagion. Troops were often gathered together from remote parts of the fledgling nation and placed into crowded camps, mingling with local civilian populations, which expanded variola transmission even further into vulnerable populations.[9] Washington proclaimed smallpox to be his "most dangerous enemy," and by 1777 he had all his soldiers variolated before beginning new military operations. In doing so, Washington fulfilled the ethical responsibility of ensuring the health of his military personnel, which in turn served to fulfill his professional responsibility as commander of a military force to preserve the nation. However, a public unfamiliar with the stakes or conditions weighing on this choice criticized Washington's actions (Figure 32-1).

Advances in military medicine and hygiene developed through experiences gained in battlefield medicine during the American Civil War were adapted as standards of medical care during the latter part of the 19th century. New medical schools such as Johns Hopkins sought advice about the most advanced patient care facilities, medical practices, and medical treatment lessons learned on the battlefield. The most direct evidence of the influence of military medicine on standard medical care practice is provided by John Shaw Billings.[10] While serving in the office of the Army surgeon general, he designed the Johns Hopkins Hospital building, applying concepts he learned about the importance of hygiene, light, and ventilation while evaluating medical care in Civil War field hospitals. Billings also created an indexing system for medical publications that was used for the Army surgeon general's library and became the nidus of the National Library of Medicine. The Welch Medical Library at the Johns Hopkins University School of Medicine adopted this same system. Additionally, the Army ambulance system was developed during the Civil War because removing injured soldiers to field hospitals had a better outcome than treating soldiers in the field. Furthermore, soldiers suffering war wounds frequently died from infection. This lesson was not lost on military physicians. As the end of the war neared, the fledgling science of bacteriology and epidemiology became hot topics of battlefield military medical research. Surgical techniques and use of anesthesia and antiseptics became commonplace during the Civil War.[11–13]

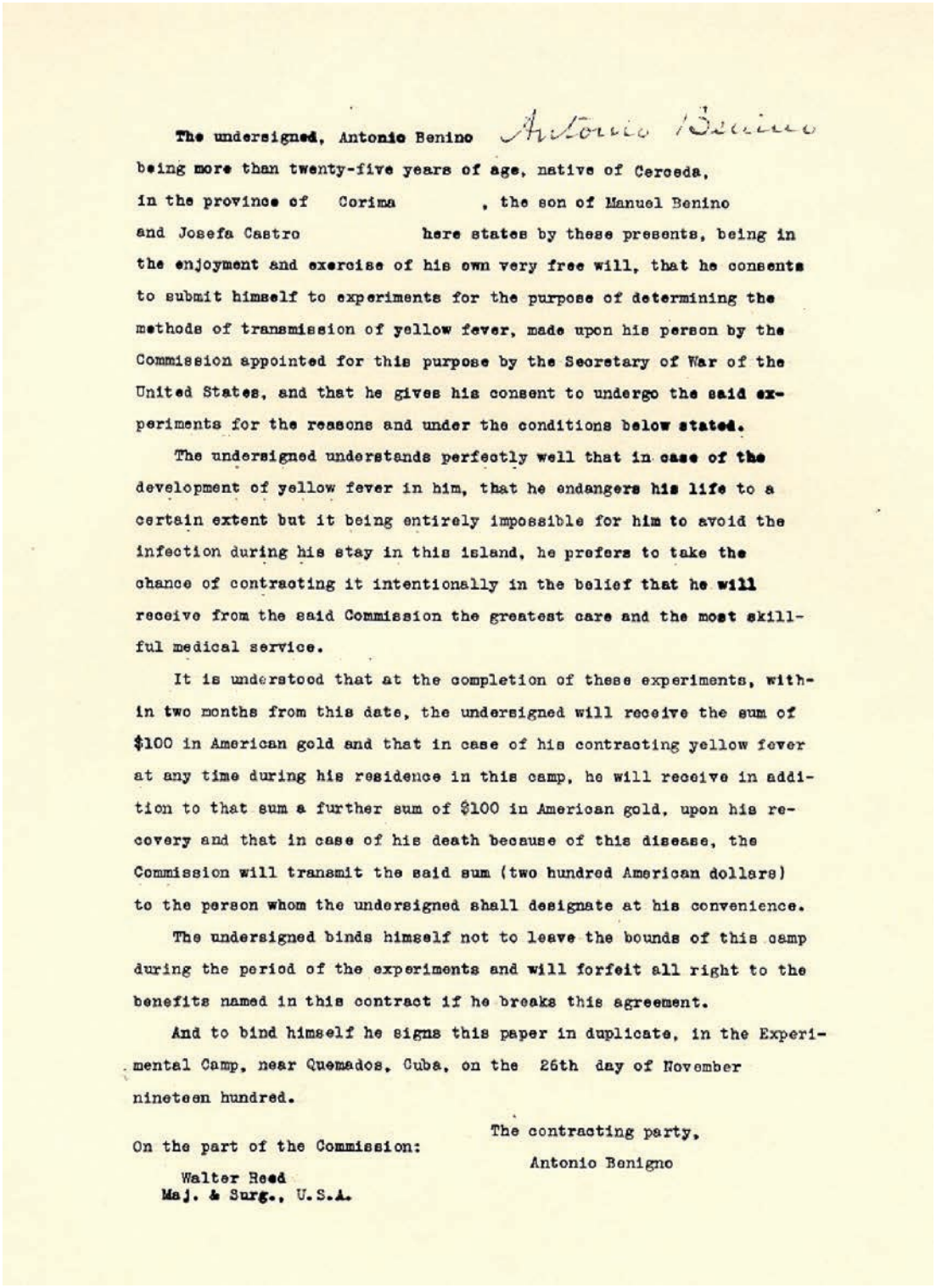


Figure 32-1. George Cruikshank, Vaccination against Small Pox or Mercenary and Merciless spreaders of Death and Devastation driven out of Society! London, England: SW Fores, 1808. General George Washington was strongly criticized in the press because of the risks and his decision to go ahead with forced variolation despite concerns. A political cartoon, published in the 1800s, shows how critically forced variolation was seen by the public despite the Army's intent to benefit its soldiers.

The Civil War was also a testing ground for medical education. One lesson learned from the war was that many who served as military physicians did not have the skills needed to save lives in the battlefield. So the Army created its own medical school at what later became the old Walter Reed Army Institute of Research building. Those who created this school liked the training being done at Johns Hopkins, where some later became faculty. Later, civilian hospitals adopted the same surgical techniques and treatment methods. Johns Hopkins Medical School created new academic standards not found at "proprietary" medical schools. Thus, with the help and influence of military medical experience, Johns Hopkins set the stage for medical treatment in the modern era.

Surgeon General George Sternberg, who had been trained as a bacteriologist at Johns Hopkins Medical School, appointed Major Walter Reed, another Johns Hopkins medical trainee, to the Yellow Fever Commission in 1900. Reed used "informed consent" statements when he recruited volunteer subjects from among soldiers and civilians during the occupation of Cuba at the end of the Spanish-American War, and those statements could be considered "personal service contracts" (Figure 32-2). These documents clearly communicated the risks and benefits of participation, described the purpose of the study, provided a general timeline for participation, and stated that compensation and

a



The undersigned, Antonio Benino Antonio Benino being more than twenty-five years of age, native of Cereeda, in the province of Corima , the son of Manuel Benino and Josefa Castro here states by these presents, being in the enjoyment and exercise of his own very free will, that he consents to submit himself to experiments for the purpose of determining the methods of transmission of yellow fever, made upon his person by the Commission appointed for this purpose by the Secretary of War of the United States, and that he gives his consent to undergo the said experiments for the reasons and under the conditions below stated.

The undersigned understands perfectly well that in case of the development of yellow fever in him, that he endangers his life to a certain extent but it being entirely impossible for him to avoid the infection during his stay in this island, he prefers to take the chance of contracting it intentionally in the belief that he will receive from the said Commission the greatest care and the most skillful medical service.

It is understood that at the completion of these experiments, within two months from this date, the undersigned will receive the sum of $100 in American gold and that in case of his contracting yellow fever at any time during his residence in this camp, he will receive in addition to that sum a further sum of $100 in American gold, upon his recovery and that in case of his death because of this disease, the Commission will transmit the said sum (two hundred American dollars) to the person whom the undersigned shall designate at his convenience.

The undersigned binds himself not to leave the bounds of this camp during the period of the experiments and will forfeit all right to the benefits named in this contract if he breaks this agreement.

And to bind himself he signs this paper in duplicate, in the Experimental Camp, near Quemados, Cuba, on the 26th day of November nineteen hundred.

The contracting party,
Antonio Benigno

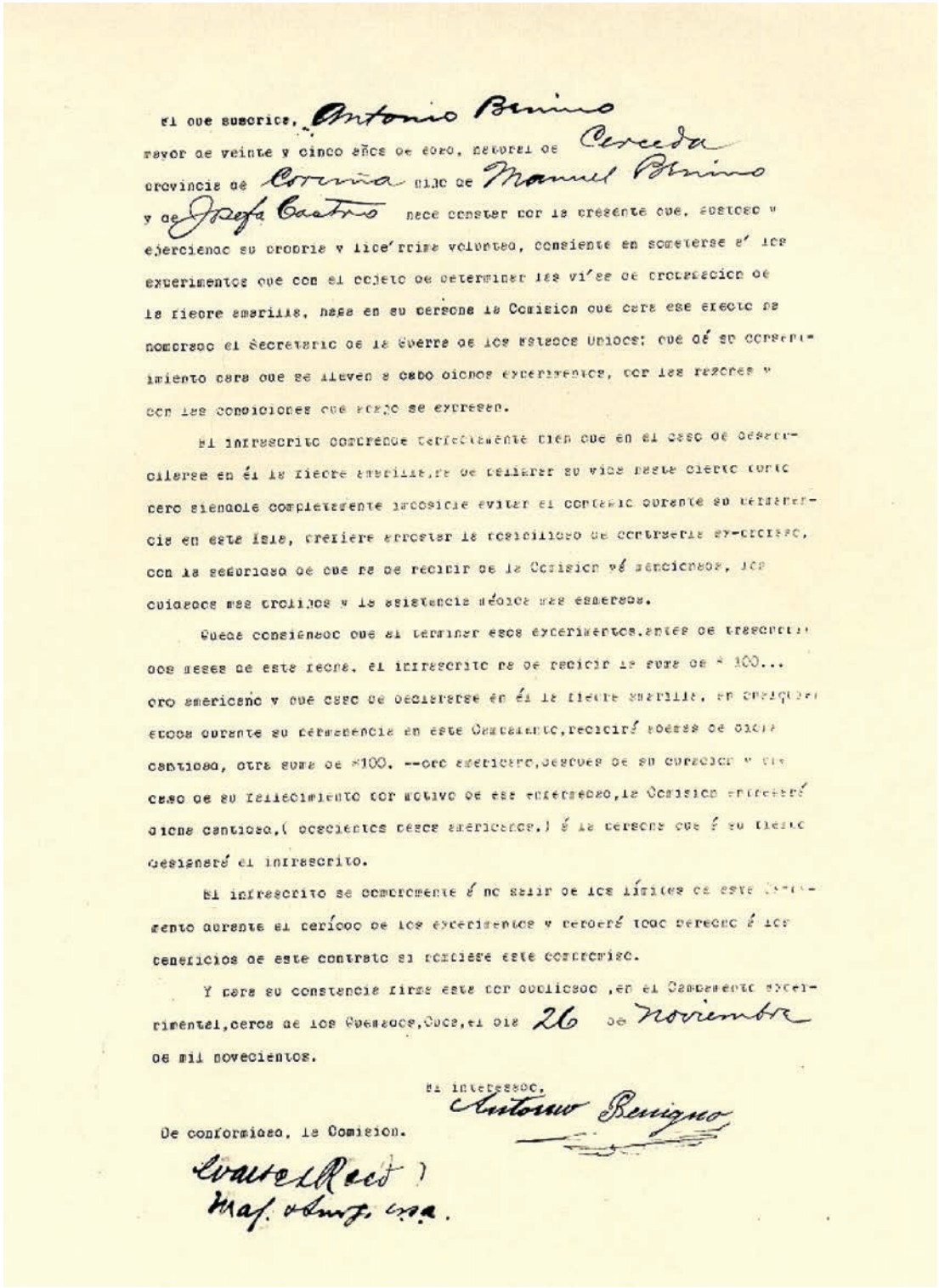
On the part of the Commission:
Walter Reed
Maj. & Surg., U.S.A.

Figure 32-2. (**a**) English translation of the yellow fever informed consent document. (**b**) Spanish version of the yellow fever informed consent documents. Major Walter Reed, who was appointed to the Yellow Fever Commission in 1900, used "informed consent" statements when he recruited volunteer subjects from among soldiers and civilians during the occupation of Cuba at the end of the Spanish-American War, which could be considered "personal service contracts." However, these

(**Figure 32-2** *continues*)

Figure 32-2 *continued*

b



El que suscribe, Antonio Benino
mayor de veinte y cinco años de edad, natural de Cerceda
provincia de Coruña hijo de Manuel Benino
y de Josefa Castro hace constar por la presente que, gustoso y
ejerciendo su propia y libérrima voluntad, consiente en someterse a' los
experimentos que con el objeto de determinar las vías de contagio de
la fiebre amarilla, haga en su persona la Comisión que cerca ese efecto se
nombrase el Secretario de la Guerra de los Estados Unidos; que de su cumplimiento cara que se lleven a cabo dichos experimentos, con las razones y
con las condiciones que luego se expresan.

El infrascrito comprende perfectamente bien que en el caso de desarrollarse en él la fiebre amarilla,se de perderse su vida hasta cierto punto
pero siendole completamente imposible evitar el contagio durante su permanencia en este lugar, prefiere arrostar la peligrosa de contraerla ex-profeso,
con la seguridad de que se de recibir de la Comisión yá mencionada, los
cuidados mas cruditos y la asistencia médica mas esmerada.

Queda consistendo que al terminar esos experimentos,antes de trascurrir
dos meses de este fecha, el infrascrito se de recibir la suma de $ 100...
oro americano y que caso de declararse en él la fiebre amarilla, en cualquier
época durante su permanencia en este Campamento,recibirá poderes de dicha
cantidad, otra suma de $100. --oro americano,después de su curación y en
caso de su fallecimiento con motivo de ese contagio,la Comisión entregará
dicha cantidad,(doscientos pesos americanos,) á la persona que á su fallecimiento designará el infrascrito.

El infrascrito se compromete á no salir de los límites de este campamento durante el período de los experimentos y perderá todos derecho á los
beneficios de este contrato si violase este compromiso.

Y para su constancia firma este por duplicado ,en el Campamento experimental,cerca de los Quemados,Cuba,el día 26 de noviembre
de mil novecientos.

El interesado,
Antonio Benigno

De conformidad, la Comision.
Walter Reed
Maj. Surg. U.S.A.

documents clearly communicated the risks and benefits of participation, described the purpose of the study, provided a general timeline for participation, and stated that compensation and medical care would be provided. All of these are standard elements required in informed consent forms provided to research participants today.
Documents: Courtesy of Historical Collections and Services, Claude Moore Health Sciences Library, University of Virginia, Charlottesville, Virginia.

medical care would be provided. All of these are standard elements required in informed consent forms provided to research participants today. Even if the yellow fever statements did not directly influence the creation of other military or civilian informed consent documents, it is at least plausible to claim that documentation of informed consent from research participants in the military predates the practice in civilian medicine.

Biodefense, Ethics, and Research in the 20th Century

Ethical issues surrounding informed consent continued into the 20th century. At the same time, the importance of strategic research was emphasized, which influenced the growth of epidemiological and infectious disease research. A 1925 Army regulation (AR) promoting infectious disease research noted that "volunteers" should be used in "experimental" research.[14] In 1932, the Secretary of the Navy granted permission for experiments with divers, provided they were "informed volunteers."[15]

The importance of strategic medical research was not unwarranted. In 1939, Japanese scientists attempted to obtain virulent strains of yellow fever virus from Rockefeller University. Vigilant scientists thwarted the attempt, but it did not take long before the threat of biological weaponry reached the War Department. In 1941, Secretary of War Henry L Stimson wrote to Frank B Jewett, president of the National Academy of Sciences, and asked him to appoint a committee to recommend actions. He wrote, "Because of the dangers that might confront this country from potential enemies employing what may be broadly described as biological warfare, it seems advisable that investigations be initiated to survey the present situation and the future possibilities."[16] In the summer of 1942, the War Research Service was established, under George W Merck, Jr, in the civilian Federal Security Agency to begin development of the US biological warfare program with offensive and defensive objectives. On October 9, 1942, the full committee of the War Research Service endorsed the chairman's statement on the use of humans in research:

> The use of human experimentation is not only desirable, but necessary in the study of many of the problems of war medicine which confront us. When any risks are involved, only volunteers should be utilized as subjects, and these only after the risks have been fully explained and after signed consent statements have been obtained which shall prove that the volunteer offered his services with full knowledge and that claims for damage will be waived. An accurate record should be kept of the terms in which the risks involved were described.[17]

Despite the War Research Service's ethical commitment to adequately inform subjects of the risks involved in research, the statement includes an assertion of waiver of rights that is now considered unethical to include in military informed consent documents. The War Research Service also supported other experiments performed by civilian scientists that involved subjects whose capacity to give valid consent to participate was doubtful, including institutionalized people with cognitive disabilities.

Meanwhile, military involvement in the development of infectious diseases research was advancing. One of the military's clear successes was the progress it made against acute respiratory disease. Because of crowded living conditions and other physical stresses, acute respiratory disease had consistently been a cause of morbidity and mortality among soldiers and an increasing economic liability for the military. In the early 1950s, military researchers under Maurice Hilleman at the Walter Reed Army Institute of Research identified seven distinct types of adenoviruses and created vaccines against them, a classic example of a quick, successful development by the military of medical countermeasures.

As the medical research community began preparing for biological threat and committing resources and time to attendant research, the undercurrent of doubts among human subjects research continued. It was not until Nazi and Japanese war crimes became public that human subjects research issues came to the forefront of the dialogue on the role and value of science in society. Dr Andrew Ivy compiled 10 conditions that must be met for research involving human subjects for the Nuremberg Tribunal in December 1946. This document, now famously referred to as the Nuremberg Code, was part of the Tribunal outcomes. In 1947, the Nuremberg Code was published in response to widespread knowledge of Nazi atrocities, including the unethical and traumatizing practices of Nazi doctors. The Nuremberg Code provided a clear statement of the ethical conditions to be met for humans as medical research subjects (Exhibit 32-1).

The DoD adopted all of the elements of the Nuremberg Code verbatim and added a prisoner-of-war provision.[18] The Army included the code in directive Cs-385, which required that informed consent must be in writing, excluded prisoners of war from participation, and included a method for DoD compensation for research-related injuries sustained by participants. In 1962, Cs-385 became AR 70-25, Use of Volunteers as Subjects of Research,[19] which regulated Army research until 1983.

In 1952, the Armed Forces Medical Policy Council noted that nonpathogenic biological warfare simulations conducted at Fort Detrick (formerly known as

EXHIBIT 32-1

THE NUREMBERG CODE (1947)

1. The voluntary consent of the human subjects is absolutely essential.

This means that the person involved should have legal capacity to give consent; should be so situated as to be able to exercise free power of choice, without the intervention of any element of force, fraud, deceit, duress, overreaching, or other ulterior form of constraint or coercion; and should have sufficient knowledge and comprehension of the elements of the subject matter involved as to enable him to make an understanding and enlightened decision. This latter element requires that before the acceptance of an affirmative decision by the experimental subject there should be made known to him the nature, duration, and purpose of the experiment; the method and means by which it is to be conducted; all inconveniences and hazards reasonably to be expected; and the effects upon his health or person which may possibly come from his participation in the experiment. The duty and responsibility for ascertaining the quality of the consent rests upon each individual who initiates, directs or engages in the experiment. It is a personal duty and responsibility which may not be delegated to another with impunity.

2. The experiment should be such as to yield fruitful results for the good of society, unprocurable by other methods or means of study, and not random and unnecessary in nature.

3. The experiment should be so designed and based on the results of animal experimentation and a knowledge of the natural history of the disease or other problem under study that the anticipated results will justify the performance of the experiment.

4. The experiment should be so conducted as to avoid all unnecessary physical and mental suffering and injury.

5. No experiments should be conducted where there is an a priori reason to believe that death or disabling injury will occur; except, perhaps, in those experiments where the experimental physicians also serve as subjects.*

6. The degree of risk to be taken should never exceed that determined by the humanitarian importance of the problem to be solved by the experiment.

7. Proper preparations should be made and adequate facilities provided to protect the experimental subject against even remote possibilities of injury, disability, or death.

8. The experiments should be conducted only by scientifically qualified persons. The highest degree of skill and care should be required through all stages of the experiment of those who conduct or engage in the experiment.

9. During the course of the experiment the human subject should be at liberty to bring the experiment to an end if he has reached the physical or mental state where continuation of the experiment seems to him to be impossible.

10. During the course of the experiment the scientist in charge must be prepared to terminate the experiment at any stage, if he has probable cause to believe, in the exercise of the good faith, superior skill, and careful judgment required of him, that a continuation of the experiment is likely to result in injury, disability, or death to the experimental subject.

*The self-experimentation clause of item 5 was omitted from the Wilson Memorandum and subsequent directives and regulations such as Cs-385 and AR 70-25 because it would be irresponsible for the person whose knowledge was essential for the safety and welfare of subjects to render himself incapacitated by taking the test agent along with his subjects.

Note: The Nuremberg military tribunal's decision in the case of the *United States v Karl Brandt et al* includes what is now called the Nuremberg Code, a 10-point statement delimiting permissible medical experimentation on human subjects. According to this statement, human experimentation is justified only if the results benefit society, and only if carried out in accord with basic principles that "satisfy moral, ethical and legal concepts."

Data source: Permissible medical experiments. In: *Trials of War Criminals before the Nuremberg Military Tribunals under Control Council Law No. 10*. Vol 2. Washington, DC: US Government Printing Office; 1946–1949.

Camp Detrick before 1956) and at various locations across the United States showed that the population was vulnerable to biological attack. Additionally, experiments with virulent disease agents in animal models attested to the incapacitating and lethal effects of these agents when delivered as weapons. However, there was doubt among the council members that extrapolation of animal data to humans was valid, and human studies appeared necessary. Ad hoc meetings of scientists, Armed Forces Epidemiology Board advisors, and military leaders occurred at Fort Detrick during the spring of 1953.[20,21] Thorough consideration of

the ethical and legal basis for human subjects research resulted in the design of several prototype research protocols and creation of the US Army Medical Unit (Figures 32-3 and 32-4). This unit heavily invested in animal experimentation but aimed at modeling human infectious diseases to study pathogenesis and response to vaccines and therapeutics. Later, the US Army Medical Unit for offensive biological warfare was discontinued, and the US Army Medical Research Institute of Infectious Diseases (USAMRIID) was established in 1969 to conduct only defensive research and development of countermeasures to select agent infections.

In 1954, military research studies using human participants began in a program called CD-22 (Camp Detrick–22) that included soldier participants in a project called Operation Whitecoat. The participants were mainly conscientious objectors who were Seventh-day Adventists trained as Army medics. The program was designed to determine the extent to which humans are susceptible to infection with biological warfare agents. The soldier participants were exposed to actual disease agents such as those causing Q fever and tularemia to understand how these illnesses affected the body and to determine indices of human vulnerability that may be used to design clinical efficacy studies. In keeping with the charge in the Nuremberg Code to protect study participants, the US Army Medical Unit, under the direction of the Army surgeon general, carefully managed the project. Throughout the program's history from 1954 to 1973, no fatalities or long-term injuries occurred among Operation Whitecoat volunteers.

Operation Whitecoat serves as a morally praiseworthy model for the conduct of biodefense research involving human subjects. The process of informed consent was successfully implemented from the inception of Operation Whitecoat. Each medical investigator prepared a protocol that was extensively reviewed and modified to comply with each of the elements of the Nuremberg Code. After a committee determined whether ethical requirements and scientific validity were met, Army officials approved the protocol. Then potential volunteers were briefed as a group regarding the approved protocol, and they attended a project interview with the medical investigator in which the potential volunteers could ask questions about the study. Informed consent documents (Figure 32-5) were signed after an obligatory waiting period that ranged from 24 hours to 4 weeks, depending on the risk involved in the study. Volunteers were encouraged to discuss the study with family members, clergy, and personal physicians before making a final decision. By allowing volunteers sufficient time and opportunity to ask questions about risks, potential benefits, and the

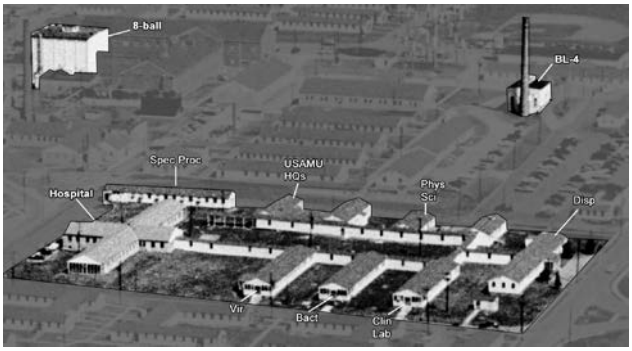
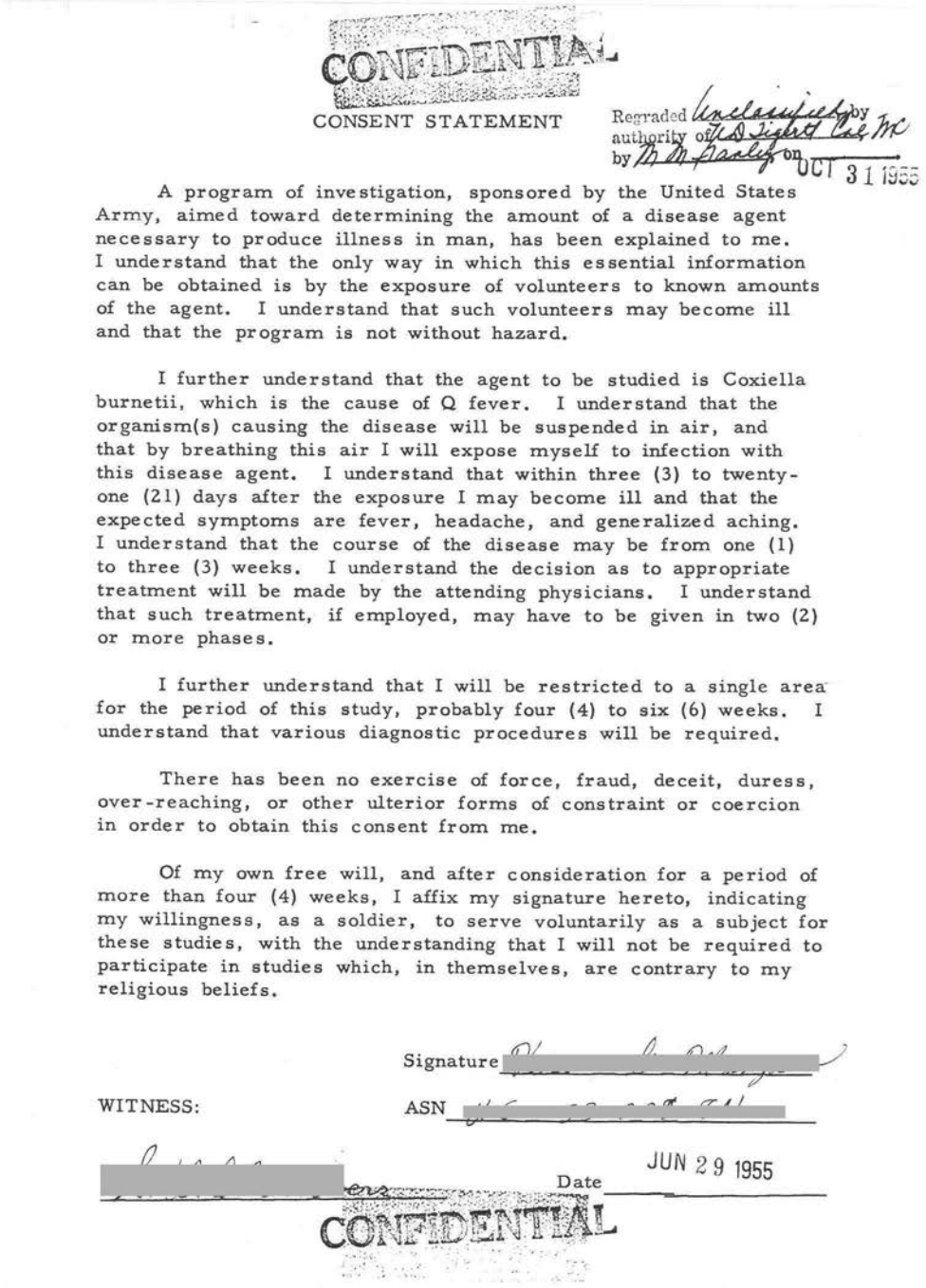


Figure 32-3. Aerial photograph of Fort Detrick, 1958. The US Army Medical Unit was assembled from existing Fort Detrick components concerned with occupational health and safety, the dispensary, and a small hospital referred to as Ward 200 of Walter Reed Army Medical Center. These components originated under separate Army commands, yet they formed an integrated, functional unit.
Photograph: Courtesy of the Department of the Army.

conduct of the study, this multistage informed consent process ensured that participation was voluntary. Soldiers were told that their participation in the research was not compulsory. Approximately 20% of those soldiers approached for participation in Operation Whitecoat declined. Review of Operation Whitecoat records of interviews with many of the volunteers and investigators revealed that the researchers informed



Figure 32-4. The US Army Medical Unit at Fort Detrick, under Colonel William Tigertt (*center*) was staffed with personnel drawn from the US Army, Navy, Air Force, and Public Health Service, whose assignment was given the highest national priority because of their unique expertise in infectious disease medical care, research, and epidemiology, and because of their determination to provide the Operation Whitecoat volunteers the best care and support for their safety during the trials. Photograph taken in 1957.
Photograph: Courtesy of the Department of the Army.



CONFIDENTIAL

CONSENT STATEMENT

Regraded Unclassified by authority of U.S. Light Col. MC by M. M. Bailey on OCT 31 1955

A program of investigation, sponsored by the United States Army, aimed toward determining the amount of a disease agent necessary to produce illness in man, has been explained to me. I understand that the only way in which this essential information can be obtained is by the exposure of volunteers to known amounts of the agent. I understand that such volunteers may become ill and that the program is not without hazard.

I further understand that the agent to be studied is Coxiella burnetii, which is the cause of Q fever. I understand that the organism(s) causing the disease will be suspended in air, and that by breathing this air I will expose myself to infection with this disease agent. I understand that within three (3) to twenty-one (21) days after the exposure I may become ill and that the expected symptoms are fever, headache, and generalized aching. I understand that the course of the disease may be from one (1) to three (3) weeks. I understand the decision as to appropriate treatment will be made by the attending physicians. I understand that such treatment, if employed, may have to be given in two (2) or more phases.

I further understand that I will be restricted to a single area for the period of this study, probably four (4) to six (6) weeks. I understand that various diagnostic procedures will be required.

There has been no exercise of force, fraud, deceit, duress, over-reaching, or other ulterior forms of constraint or coercion in order to obtain this consent from me.

Of my own free will, and after consideration for a period of more than four (4) weeks, I affix my signature hereto, indicating my willingness, as a soldier, to serve voluntarily as a subject for these studies, with the understanding that I will not be required to participate in studies which, in themselves, are contrary to my religious beliefs.

Signature [redacted]

WITNESS: ASN [redacted]

[redacted] Date JUN 29 1955

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Figure 32-5. Early (1955) informed consent used for one of the Camp Detrick-22 Operation Whitecoat experiments. Document: Courtesy of Medical Records Archives, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

participants that the research was scientifically valid and potentially dangerous, and that any harm to the participants would be minimized.

Approximately 153 studies related to the diagnosis, prevention, and treatment of various diseases were completed during Operation Whitecoat, including research on Q fever and tularemia infections and staphylococcal enterotoxins. Vaccines to be used against Venezuelan equine encephalitis, plague, tularemia, Rocky Mountain spotted fever, and Rift Valley fever were tested for evidence of safety in humans. However, scientists conducted animal studies before human

subjects research. For instance, researchers exposed Operation Whitecoat volunteers to aerosolized Q fever organisms only after completion of animal safety and efficacy studies. The first exposure occurred on January 25, 1955, with the use of a 1-million-liter stainless steel sphere at Fort Detrick known as the "Eight Ball." This research device was designed to allow exposure of animals and humans to carefully controlled numbers of organisms by an aerosol route.

Research conducted during Operation Whitecoat also contributed to the development of equipment and procedures that established the standard for laboratory biosafety throughout the world. The ethical commitment to the safety of laboratory workers engaged with dangerous toxins, viruses, and diseases was manifested by the development of biological safety cabinets with laminar flow hoods, "hot suites" with differential air pressure to contain pathogens within the suites, decontamination procedures, prototype fermentors, incubators, refrigerated centrifuges, particle sizers, and various other types of specially fabricated laboratory equipment. Many of the techniques and systems developed at Fort Detrick to ensure worker safety while handling hazardous materials are now used in hospitals, pharmacies, and various manufacturing industries worldwide.

Operation Whitecoat was not the only example of US military involvement in human subjects research, and not all involvement in human subjects research reflects favorably on the US military. For example, the US military conducted unethical research involving LSD on uninformed human subjects from 1958 to 1964.[22] Congress enacted the National Research Act of 1974 because US Public Health Service personnel and civilian collaborators at the Tuskegee Institute violated human subjects' rights, most famously in the Tuskegee syphilis experiments.[23] This act immediately imposed rules for the protection of human subjects involved in research, requiring informed consent from subjects and review of research by IRBs. The act created the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research, which published the Belmont Report, a compilation of the principles implicit in ethical medical practices, in 1979. The commission also provided a schema for the formal review of research by standing committees. Belmont Report findings were incorporated into AR 70-25 in 1988.[19]

The ethical principles identified in the report, including the principles of respect for persons, beneficence, and justice, were compiled from a review of codes of conduct and standard medical and research ethics practices. Respect for persons refers to those practices whereby the right of individuals to make fully informed decisions is respected, and the need for protection of persons who are less able to exercise autonomy is recognized. Beneficence refers to the deliberate intention to do good and the assurance that participation in the research is more likely to result in good than in harm. Justice demands that the potential benefit and harm of the research be distributed fairly in society, which has typically been understood to mean that the research cannot solely assist or exploit any certain demographic.

In practice, these three principles yield the research requirements respectively for informed consent, risk/benefit analysis, and fair inclusion/exclusion criteria for participants. Much has been written about these principles, their flexibility and adequacy as guides, and their connection to philosophical foundations,[24–26] and they remain appreciated as a practical approach to considering actions in biomedical contexts. The principles are secular but not incompatible with religious views, and they recognize the value of human individuals and the importance of collective benefits. The principles were incorporated into all federal institutions that fund research, including the DoD, as part of this common rule. Hence, "common rule" became the catch phrase used to refer to the institution-wide incorporation of explicit ethical requirements as identified in the Belmont Report. In 2011, the DoD Directive 3216.02, more recently updated as a DoD Instruction (DoDI) 3216.02, was implemented "to establish policy and assign responsibilities for the protection of human subjects in DoD-supported programs to implement part 219 of title 32, Code of Federal Regulations (CFR) (also known and hereinafter referred to as "the Common Rule").[27]

Success in incorporating ethical principles into human subjects research in the military in the mid-20th century was complemented by numerous early achievements of military researchers of high moral character who ethically developed vaccines for a variety of infections, including yellow fever (1900), typhoid fever (1909), pneumonia (1945), hepatitis A (1945), influenza (1957), rubella (1961), adenovirus (1952–1969), and meningococcal disease (1966) without written regulations.[9]

IMPACT OF REGULATING AGENCIES ON STRATEGIC RESEARCH

The Public Readiness and Emergency Preparedness Act provides compensation to individuals for serious physical injuries or deaths from pandemic, epidemic, or security countermeasures identified in a declaration issued by the secretary pursuant to section 319F-3(b) of the Public Health Service Act (42 USC 247d-6d).[28] The emergency use authorization (EUA) program was established in 2004, when

the Project BioShield Act, among other measures, amended Section 564 of the Federal Food, Drug, and Cosmetic Act to include this provision.[29] EUA permits the FDA commissioner to authorize the use of an unapproved medical product or an unapproved use of an approved medical product during a declared emergency involving a heightened risk of attack on the public or US military forces, or a significant potential to affect national security.[30]

The evolution of regulatory bodies overseeing human subjects' research paralleled the evolution of military medical research ethics. These regulatory bodies influenced military research in positive and negative ways. In 1901 in Missouri, 13 children died of tetanus after receiving horse serum contaminated by *Clostridium tetani* for treatment of diphtheria. In 1902, Congress enacted the Biologics Control Act (the Virus-Toxin Law), which gave the federal government authority to require standards for the production of biological products, including vaccines. The act contained provisions for establishing a board (including the surgeons general of the Navy, Army, and Marine Hospital Service) with the power to create regulations for licensing vaccines and antitoxins. Thereafter, only annually licensed, inspected facilities were permitted to produce biologics. This act marked the commencement of America's federal public health policy for biologics.

The 1938 Food, Drug, and Cosmetic Act regulated biologics through mid-century. For the first time, drug production had to meet standards for safety before receiving approval for marketing. The 1944 Public Health Service Act reinforced or expanded public health policy standards in two ways: (1) it became the mechanism containing explicit regulation of biologics, and (2) it created the FDA. Under its new authority, the FDA approved the influenza vaccine, chiefly on the strength of data provided by the Army.[31]

In 1962, Congress passed the Kefauver-Harris Drug Amendments to the Food, Drug, and Cosmetic Act, which effectively launched the modern US drug regulatory system. These amendments stipulated an intense premarketing approval system, giving FDA the power to deny approval for products with safety concerns. The amendments also required proof of human efficacy for all drugs and biologics, including vaccines.

The requirement for proof of efficacy of all medical countermeasures, premised on the principle of protecting the lives and other interests of human subjects, is a responsible action. But the Kefauver-Harris Drug Amendments also categorized the only available medical countermeasures against biological weapons as unapproved new drugs requiring approvals, which created an ethical dilemma for the DoD. Compliance with the FDA regulations meant that the DoD either had to risk the deaths of human subjects in a valid clinical trial, or withhold potentially life-saving drugs or vaccines because they lacked substantial evidence of human clinical efficacy. Currently, the FDA allows for "expanded access" IND applications for unapproved new drugs that have demonstrated sufficient safety and efficacy to warrant their limited use. (The drugs and vaccines in question would all require evidence of animal efficacy, unless no animal model of human disease could be found.) Additionally, AR 70-25 [1962, 1974, and 1988, but not 1990][32] contained clauses [3c] that exempted biodefense research and testing if there was intent to benefit the research subject. To resolve this issue, the DoD sought exceptions to these new regulations by negotiating memoranda of understanding (MOU) with the FDA in 1964, 1974, and 1987. An MOU provided the FDA an assurance that the DoD would conduct clinical testing of biologics, categorized as unapproved new drugs, under FDA regulations, including requirements for human subject informed consent, IRB review, and controlled clinical trials in medical research (see 21 CFR 50 and 56).[33] The MOU states that the DoD will meet these requirements without jeopardizing responsibilities related to its mission of protecting national interests and safety. Additional MOUs between the DoD and the FDA have been established, and include the following:

- MOU 224-75-30033,[34] which "establishes the procedures to be followed regarding the investigational use of drugs, including antibiotics and biologics, and medical devices by DoD;" and
- MOU 225-07-8003[35] for "sharing of information and expertise between the Federal partners" as well as regulation AR 40-7, which governs the Army's use of investigational products.

CONFLICT BETWEEN REGULATIONS AND ETHICAL RESPONSIBILITIES

The military situation is unique. In the tension between the good of the individual and the good for the social organization, the latter justifiably holds greater weight in decision-making procedures in the military context. Members of the military have unique responsibilities, which include being fit for duty. The military organization also has responsibilities to its service members, including providing healthcare specific to the dangers encountered in deployment locations.

Department of Defense/Food and Drug Administration Memorandum of Understanding (1987)

The 1991 Persian Gulf War brought into focus the inadequacy of the 1987 MOU and the conflicts between the duties of the two agencies. The DoD's mission is to protect the interests of the United States. The DoD also recognizes its ethical responsibility to protect the health of military personnel. Thus, the DoD is doubly obligated to the mission and to service members. It is the responsibility of service members to keep themselves fit throughout the current mission and for future missions. When troops are threatened by biowarfare, in the absence of an approved biodefense product, one supported by preclinical data may be the only available option for troop protection. With a credible threat, the situation is similar to that of patients with an incurable disease who wish to try a potential remedy in advance of large clinical trials if it offers plausible expectation of some benefit. Such a product administered but proven ineffective would be analogous to sending troops to battle with faulty equipment. Such a product later proven unsafe would be analogous to friendly fire—perhaps an even more damaging situation for morale. Thus, the military requires a fine balance between necessity and caution. Proper biodefensive posture requires effective therapeutic countermeasure prophylaxis or treatment and, when appropriate, vaccination against credible threats.

Currently, vaccinations include licensed anthrax and smallpox vaccines and unlicensed vaccines for tularemia, botulism toxin poisoning, and a variety of encephalitides, including Venezuelan equine encephalitis, western equine encephalitis, and eastern equine encephalitis. Data for these unlicensed vaccines support human safety and efficacy,[36] even though efficacy has been demonstrated only in animals. Medical experts favor the use of these vaccines in protecting human beings when threat dictates. Because the vaccines are not licensed and will not—for ethical reasons—undergo the clinical efficacy trials required by FDA, they can only be used in an IND status unless testing and efficacy is demonstrated under FDA-approved Good Laboratory Practices efficacy studies animal rule.

Investigational New Drug Status of Therapeutics and Vaccines

FDA considers any administration of an investigational product to a human to constitute a clinical investigation and authorizes the administration of an investigational product only in the context of an IND, which permits clinical research trials to move forward[37] or expanded access treatment to be administered under an emergency treatment IND application in which the investigational product use would not considered research. Because the therapeutic benefit of the investigational product is unknown, FDA also requires informed consent. Administration of an investigational product requires specific and detailed recordkeeping measures. However, the recordkeeping requirements relate specifically to research, not to emergency or preventive measures connected to imminent risk of biological attacks on the battlefield. It would take exponentially longer to collect data from and perform recordkeeping for 100,000 soldiers than to merely administer an unlicensed therapeutic or vaccine for treatment or prevention purposes. The consenting process alone for 100,000 individuals receiving an investigational product would take so long that strategic combat moves, such as immediate mobilization and deployment of a unit, would be impossible. Storing informed consent documents for 100,000 soldiers, and the accompanying logistical challenge of reconsenting soldiers if new risk information emerged during deployment, would also be daunting. It has been estimated that implementing only one protocol for an investigational product may generate up to 94,000 lbs of paper records in a theater of operations, according to physicians discussing rewriting IND protocols in meetings held in 2002 and 2003 at Fort Detrick, Maryland. Furthermore, continuous data collection, as required by the FDA's Good Clinical Practices (GCPs), is unfeasible and would effectively result in noncompliance problems, such as what occurred during the Persian Gulf War. FDA regulations governing storage and distribution of INDs (21 CFR 312.57 and 59)[33] are specific and limiting, which would render some therapeutics and any immunization schedule impossible in the field.

The FDA's commitment to protecting the citizenry from the unknown effects of medical treatments has thus resulted in two legal quandaries. First, the FDA permits the use of unapproved products, including the vaccines in question, for research purposes under an IND.[38] However, the situation in war is not a research situation but it still requires the use of an unapproved product under an IND protocol unless the notice requirement is fulfilled under 10 USC 1107(a); the product is administered during the practice of medicine; or it is provided under an IND protocol approved by the FDA. Giving these products to military personnel before engagement in war for purposes of preventing disease caused by a biowarfare agent constitutes a treatment application of the product and is not considered research. No benefit is believed to accrue to an individual receiving an investigational product.

All IND protocols are clinical investigations, but not all clinical investigations are considered research. For example, off-label use of an approved product with no intention of reporting the safety and efficacy data to the FDA is not considered research under the Common Rule. However, both research and clinical investigations must receive approval by a formal IRB. Administration of investigational therapeutic or vaccination to military personnel in wartime does not constitute research, even though it is the only classification FDA permits for these unlicensed and untried vaccines. Continuing to categorize such vaccines and drugs as "investigational" also fails to inspire confidence in soldiers asked to receive the therapeutic or vaccine, even if limited evidence shows that the product is not only safe but likely efficacious based on extrapolation from animal data. The label "investigational" does not communicate the strength of the data from animal studies that supports the safety and efficacy of the product. It creates the perception that soldiers at risk of losing their lives in combat are also being used as subjects of research—or guinea pigs—despite the intent to use these products solely for the soldiers' protection.

The FDA requires informed consent from subjects receiving investigational products. Consequently, subjects have the right to decide whether they will receive the investigational drug, and soldiers must understand that they cannot be required to take investigational drug products. The requirement for informed consent is based on the Nuremberg Trial findings related to research in which benefits did not directly accrue to research participants. In the context of preventive treatment in a military conflict, the requirement for informed consent is a misapplication of a principle of clinical research ethics. Enlisted and commissioned soldiers surrender much of their autonomy in matters of choice and accept the relinquishment of autonomy as a standard of military discipline and law. Specifically, one of the rights that military personnel forsake is the discretionary authority over their medical treatment under the rules and regulations governing force health protection (FHP). FHP can be defined as all services performed, provided, or arranged by the armed services to promote, improve, conserve, or restore the mental or physical well-being of personnel. The requirement for informed consent threatens to put a divisive wedge between commander and subordinates, and such discord is counterproductive to military recruitment, retention, and mission accomplishment. One solution to this problem may be to move IND products to licensure either by obtaining FDA approval through the use of the animal efficacy rule or by BioShield EUA, with all of the attendant medical subject matter expert board review and input afforded to products going before the FDA.

In the first Persian Gulf War, the DoD was acutely concerned with protecting military personnel from harm related to biological weapons. Intelligence indicated that Iraq had not only used chemical weapons against humans in the past, but it had also manufactured and stockpiled biological weapons that were believed to be ready for use. In documents sent to the FDA regarding implementing proper biodefense in military personnel against botulism, the DoD argued that waiver of informed consent was justified because a botulism vaccine (also referred to as the pentavalent botulinum toxoid vaccine) was to be administered as protection of and not as research on military personnel. The FDA accepted this DoD argument and exempted the DoD from the data gathering and recordkeeping requirements typically required during the administration of investigational products.[39–44]

This decision had historic consequences. Some commentators characterized the FDA's accommodation of the DoD's wishes as unethical. This accusation resulted in changes in the relationship between the FDA and DoD after veterans claimed "Gulf War syndrome" injuries. Gulf War syndrome is a phrase used to capture the constellation of injury claims stemming from symptoms experienced by Gulf War veterans after the conflict, some of which have been attributed to anthrax and/or botulism vaccination.[45,46] Despite repeated high visibility detailed studies conducted by the Institute of Medicine of the National Academies of Science, no causal relation has been shown between these symptoms and receipt of either vaccine.[47–51] Most soldiers who received inoculations from the same lots of vaccine as those who claim illness did not experience any of the associated symptoms. Furthermore, the majority of claims of illness were associated with receipt of the anthrax vaccine, which was already an FDA-licensed product for inhalational and cutaneous anthrax at the time of deployment for the first Persian Gulf War, rather than the botulism vaccination, which few soldiers received. Articles that summarize long-term outcomes after receipt of multiple vaccines, including those used during the Persian Gulf War, address the safety of these vaccines.[38,52,53] But even if the existence of a causal relationship between receipt of the vaccine and the manifestations of the Gulf War syndrome is accepted, the DoD's use of the vaccines to protect the force was an ethically supportable decision. It was an ethically supportable decision first and foremost because military intelligence indicated botulism was Iraq's biological weapon of choice, which meant there was a likelihood of its use during military operations. Any use of botulism by the Iraqi forces would place American soldiers directly in harm's way, but to an extent greater than would be faced during most traditional 20th century warfare.

This scenario was one of the instigating factors that led Congress to enact 10 USC 1107(a) (notice for off-label uses required) and 10 USC 1107(f) (EUA provisions for DoD).[54] The DoD had an obligation to meet this extra threat for its soldiers' health and for the benefit of the military mission. To meet this threat as ethically as possible, subject matter experts weighed in on the risks and benefits of using the vaccine, and discussions between the DoD and FDA were held. The possibility of ill effects from the vaccine is an unintended consequence of the situation, but they could not have been known beforehand and do not alter the ethically supportable dimensions of the decision-making process, the intentions, or even the execution of the plan to vaccinate soldiers.

Summary Points

Historically Human Subjects Protections Regulations Had Been Incompatible With Department of Defense Deployments

The immediacy of war preparations and the chaos generated during military operations work against requirements of human subjects protection, including the requirement to solicit and obtain informed consent from subjects. Receipt of an IND drug must be voluntary. However, by definition, true FHP measures cannot be voluntary. The voluntary nature of FDA-regulated research can undercut the effectiveness of FHP measures, which rely on universal compliance for their efficacy. FHP measures, which are necessary for successful operations in war, are imposed to safeguard the soldiers' health. If left to the choice of individual soldiers, the health benefit to the soldier may be compromised and military success jeopardized. To mitigate this dilemma, under 10 USC 1107 a waiver may now be granted where informed consent would not be necessary, thus allowing the incorporation of unlicensed new drugs into the FHP program. Military personnel, who have ceded part of their autonomy to the government as a condition of service, are obligated to accept command-directed protective measures in the United States (immunizations are voluntary in the United Kingdom and in most European militaries). However, waiving the requirement for informed consent for receipt of an investigational product can undermine public trust and military morale. The FDA requirement for informed consent for receipt of an investigational product is mandated as necessary for research protocol approval and is premised on the idea that administration of an investigational product is for research purposes, and the safety and efficacy of the drug are unknown. The caveat to this requirement is the use of the EUAs and expanded access IND applications in support of deployments, both of which are not considered research. If countermeasures without medically significant contraindications were licensed for therapeutic purposes, this would lower the threshold for requiring informed consent. Licensure "for military use" would remove the stigma attached to use of an agent categorized as "investigational" for research purposes and mitigate the need for the use of investigational products along with the potential of failure in meeting the stringent regulatory requirements established for human subjects protection.

Realities of Deployment Conflict With Food and Drug Administration Regulations and Guidance

GCP data requirements support new product license applications, but GCP data collection does not serve the purposes of DoD military use of selected (unlicensed) medical products. The FDA enforces clinical data collection on IND products as a function of stringent protection of research integrity. Shortfalls in data management, such as missing data, missing vials, or missing forms, are inevitable during expediencies of real-time deployment and the exigencies of warfare, making it difficult for the DoD to meet FDA requirements. Unanticipated or unavoidable protocol violations and deviations inevitably occur, even under ideal investigational circumstances, and even when researchers fully intend to strictly follow GCP requirements. Unforeseen circumstances encountered in war are unavoidable. Scientific misconduct, then, may be suspected when the realities of deployment work against traditional scripted research strategies. Ultimately, force protection—not research—is the primary purpose of the military use of these countermeasures.

OPTIONS FOR FULFILLING MISSION AND ETHICAL RESPONSIBILITIES TO MILITARY PERSONNEL

Option 1: Continue to Use Investigational New Drug Products Without Full Compliance

Regardless of a presidential waver of informed consent, the DoD cannot use investigational products without many instances of noncompliance with GCP unless the needed product is approved under an EUA. However, the EUA was not available to DoD during the deployments to Desert Storm in 1991 and Bosnia in 1992, and the return deployment to Iraq in 2003. Therefore, it was predictable that serious and continuing noncompliance would result from use of

IND products in a dynamic battlefield environment. GCP conflict with the requirements of countermeasure use during wartime, as seen during the first Persian Gulf War. The ethical responsibility of the DoD to protect soldier health and welfare does not commit the DoD to creating marketable products. However, if the data are gathered on these investigational products during wartime with the intention of increasing product knowledge, then GCP restrictions may need to be adaptable and flexible for wartime military use because of the inherent limitations imposed by military operations. These changes would permit the DoD to contribute to research by adding to the data gathered before bringing these investigational products to market. DoD can choose to move forward with a particular investigational product while doing its best to use the product according to FDA requirements, including adhering to GCP when practical.

Problems

Any relaxation of FDA standards could facilitate an impression of abuse of power by the DoD. Accusations of product approvals without sufficient consideration of safety issues could result in legal and economic fallout for the federal government. Most importantly, relaxing these standards, which the FDA has put in place to protect citizens, could result in a patient's injury or death.

Option 2: Negotiate for Accelerated Licensure

The DoD can negotiate with the FDA for assistance in hastening licensure of products required in contingencies or for FHP. If the DoD negotiates directly with the FDA, then drugs and vaccines could be given without the burden of research format and documentation. Epidemiological follow-up—not case report forms—would determine benefit, and decisions to retain or withdraw approval could be based on epidemiological analyses. The DoD could ask the FDA to waive investigational drug requirements that cannot be practicably met in specific cases. Finally, there are now several mechanisms by which investigational products may be used by the DoD for FHP to include 10 USC 1107(a) notice provision, presidential wavers to advanced informed consent, EUA, fast-tract product approval, accelerated approval, breakthrough therapy designation, and orphan drug status.[55]

Problems

The potential for DoD abuse of such power, or even the perception of abuse of such powers, will always be present. In addition, applications for FDA licensure must originate from the patent holder, not the DoD.

Option 3: Institute Waiver of Informed Consent

Although considered a necessary condition for research to be ethical, the requirements for obtaining informed consent (21 CFR 50.20-.27, 32 CFR 219.116-.117, 45 CFR 46.116-.117)[33,56,57] are not absolute. If informed consent is unfeasible or contrary to the best interests of recipients (21 CFR 50),[33] such as in emergency situations or where the subject cannot give informed consent because of a medical condition and no representative for the subject can be found, the requirement can be waived. Executive Order 13139 and the Strom Thurmond National Defense Authorization Act of 1999 give the president of the United States the power to waive the requirement for informed consent for the administration of an unlicensed product to military personnel in connection with their participation in a particular operation.[58] The requirements are a formal request from the secretary of defense for such a waiver, based on evidence of safety and efficacy weighed against medical risks, and the requirement that a duly constituted IRB must approve the waiver, recordkeeping capabilities, and the information to be distributed to soldiers before receipt of the drug or vaccine.

One might argue that there is no need for a waiver of informed consent. If a soldier refuses receipt of a particular unlicensed product, he or she can be replaced by another soldier who is willing. But one does not have to search far for a scenario where waiver of informed consent might be warranted. The present day worries over recruitment and retention reflect this situation.

Problems

Some existing regulations conflict with the president's power to waive informed consent requirements for military personnel, including conflicts and limitations posed by Title 10 USC Section 980 (10 USC 980),[59,60] AR 70-25.[19] Title 10 USC 980 reads as follows:

Funds appropriated to the Department of Defense may not be used for research involving a human being as an experimental subject unless (1) the informed consent of the subject is obtained in advance; or (2) in the case of research intended to be beneficial to the subject, the informed consent of the subject or a legal representative of the subject is obtained in advance.[35]

10 USC 980 contains no provision for waiver of the requirement for informed consent, not even for the president, and neither of its two conditions for waiving the requirement would be met by a presidential waiver. However, DoDI 3126.02 allows for a waiver of

the advance informed consent provision of 10 USC 980 for research conducted under 21 CFR 50.24, Exception from Informed Consent for Emergency Research.

Chapter 3, section 1, paragraph (f) of AR 70-25 states that "voluntary consent of the human subject is essential. Military personnel are not subject to punishment under the Uniform Code of Military Justice for choosing not to take part as human subjects. No administrative sanctions will be taken against military or civilian personnel for choosing not to participate as human subjects."[17] Therefore, when the DoD and USC regulations are compared and interpreted including DoDI 6200.02, Section 5.2.4 medical products for FHP, DoDI 3216.02, subject research definitions, 10 USC 980 (informed consent for human subject research), and 10 USC 1107 (f) (presidential consent waiver in specific military operations for FHP), these regulations are in agreement. Thus, the DoD and USC regulations are not in conflict because 10 USC 980 (informed consent for human subject research) and 10 USC 1107 (f) (presidential waiver) are not in conflict because the latter applies to a waiver for a specific military operation, which is FHP and not human subject research.

An additional problem with presidential waiver of informed consent is the requirement that such a waiver be posted for public review in the *Federal Register*. This requirement makes operational secrecy impossible, especially given the length of time some vaccines require to elicit adequate titers in recipients.

Also, public perception is a looming issue. If the requirement for informed consent is waived—even by the president—public backlash is not likely to be quiet or short lived. Public awareness of research subject abuse has grown, and the public is aware that informed consent is essential for the ethical use of products for which the FDA cannot claim knowledge of safety and efficacy. Public outrage directed at the military, and the subsequent erosion of trust between the government and the governed, is a risk that also must be considered.

CURRENT MOVEMENTS IN THE REGULATORY ENVIRONMENT

Further restricting the ability of the DoD to properly protect military personnel with vaccines with preclinical evidence of efficacy would not be the best solution to this legal and ethical dilemma. If the DoD were to eschew unlicensed products and the IND issue entirely, an argument could be made that military personnel would be at greater risk from infectious agents. However, several options are available to address this issue,[34] some of which have seen dialogue or attention in the form of legislation.

The Public Health Security and Bioterrorism Preparedness and Response Act of 2002

The Public Health Security and Bioterrorism Preparedness and Response Act of 2002, also called the Bioterrorism Act and the Guidance for Industry: Expedited Programs for Serious Conditions – Drugs and Biologics,[60] contains several provisions to facilitate approval of vaccines and other priority countermeasures eligible for accelerated approval, clearance, or licensing. Title II of the act also contains the kernel of what is known as biosurety, which is a combination of biosafety, security, and personal reliability needed to safeguard select biological agents and toxins that could potentially be used in bioterrorism. Finally, this act approved the animal efficacy rule.[61] The Guidance for Industry document outlines expedited programs to include fast track, breakthrough therapy, accelerated approval, and priority review designations.

21 Code of Federal Regulations 314 Subpart I: The Animal Efficacy Rule

Another regulatory response that reflects a positive move toward reducing conflicts in responsibilities between the FDA and DoD was the creation of an animal efficacy rule. A draft animal efficacy rule was prepared by the FDA commissioner's office and had been published for public comment 2 years before the terrorist attacks in fall 2001. The FDA recognized the acute need for an animal efficacy rule that would help make certain essential new pharmaceutical products available much sooner. These products, such as current IND vaccines, cannot be safely or ethically tested for effectiveness in humans because of the nature of the illnesses they are designed to treat.

The FDA amended its new drug and biological product regulations so that certain human drugs and biologics intended to relieve or prevent serious or life-threatening conditions may be approved for marketing based on evidence of effectiveness from appropriate animal studies when human efficacy studies are not ethical or feasible. The FDA took this action because it recognized the need for adequate medical responses to protect or treat individuals exposed to lethal or permanently disabling toxic substances or organisms. This new rule, part of FDA's effort to help improve the nation's ability to respond to emergencies, including terrorist events, applies when adequate and well-controlled clinical studies in humans cannot be ethically conducted because the studies would involve

administering a potentially lethal or permanently disabling toxic substance or organism to healthy human volunteers.

Under the new rule, certain new drug and biological products used to reduce or prevent the toxicity of chemical, biological, radiological, or nuclear substances may be approved for use in humans based on evidence of effectiveness derived only from appropriate animal studies and any additional supporting data. Products evaluated for effectiveness under the rule will be evaluated for safety under preexisting requirements for establishing the safety of new drug and biological products. The FDA proposed this new regulation on October 5, 1999, and the rule took effect on June 30, 2002. The advent of the animal efficacy rule shows the importance of animals in finding safe and effective countermeasures to the various toxic biological, chemical, radiological, and nuclear threats.

Using animal surrogates to prove clinical efficacy is not a perfect solution, even though it is the only ethical and moral solution in the case of drugs and vaccines aimed at mitigating biowarfare or bioterrorism threats. Additionally, use of animals in infectious disease research presents its own ethical and moral dilemma. Intentional infection of animal research subjects with deadly diseases requires strong consideration of the research harm versus benefit analysis by the institutional animal care and use committee as well as development of species and disease-specific humane early endpoints.[62] Use of analgesics and humane early endpoints in animal studies is not always standardized among infectious disease researchers, potentially making comparison of research data difficult. Other challenges include conduct of research under animal biosafety level 3-4 and good laboratory practices conditions, development of adequate animal models, and gaining clear FDA guidance on research design and animal manipulation methods.

To improve the validity of animal efficacy studies as models of human clinical efficacy, it is important to be rigorous in searches for the most optimal model that accurately mimics human disease. It is also necessary to draw precise comparisons between immune responses and drug kinetics in the animal surrogate and analogous responses in patients who participate in product safety but not clinical efficacy studies. Furthermore, because drugs approved by the animal efficacy rule may still not be "proven" efficacious in humans, postmarketing epidemiological studies are necessary to monitor outcomes. Finally, some diseases, such as dengue and smallpox, only affect human beings and do not affect animals. If animal efficacy data cannot be produced for a disease, the implication is that no vaccine could be created or used in human beings, which hardly seems a fitting solution. Testing of countermeasures against disease surrogates (closely related diseases) that do have animal models or the use of in vitro tissue culture assays systems may be the only alternatives to evaluating some diseases that lack a suitable animal model.

BioShield Act of 2004

Project BioShield was designed to speed the development and availability of medical countermeasures in response to bioweapons threats by accelerating and streamlining government research on countermeasures, creating incentives for private companies to develop countermeasures for inclusion in a national stockpile, and giving the government the ability to make these products quickly and widely available in a public health emergency to protect citizens from an attack using an unmodified select agent.

The BioShield Act of 2004 created permanent funding for the procurement of medical countermeasures and gave the federal government the power to purchase available vaccines. The FDA and Department of Health and Human Services are tasked not only with determining that new vaccines and treatment measures are safe and efficacious, but also with the responsibility of making promising vaccines and treatment measures expeditiously available for emergency situations. The FDA Emergency Use Authorization for Promising Medical Countermeasures provides one of the best ways of getting such products to those who might need them most, including military personnel. The legislation also requires the secretary of the Department of Health and Human Services to approve such emergency use measures, with the added requirement of FDA expert opinion that the benefits of the vaccine or treatment outweigh the risks involved in its application. Just such an emergency use of anthrax vaccine adsorbed (Biothrax, BioPort Corporation, Lansing, MI) was approved by Health and Human Services Secretary Tommy G Thompson on January 14, 2005, authorizing its emergency use.

Under DoDI 1107a (emergency use products), a waiver by the president would permit the use of a product in the times of an emergency:

> Waiver by the President. (1) In the case of the administration of a product authorized for emergency use under section 564 of the Federal Food, Drug, and Cosmetic Act to members of the armed forces, the condition described in section 564(e)(1)(ii)(III) of such act and required under paragraph (1)(A) or (2)(A) of such section 564(e), designated to ensure that individuals are informed of an option to accept or refuse administration of a product may be waived only by the President only if the President determines in writing that complying with such requirement is not in the interest of national security. (2) The waiver

> authority provided in paragraph (1) shall not be construed to apply to any case other than a case in which an individual is required to be informed of an option to accept or refuse administration of a particular product by reason of a determination by the Secretary of Health and Human Services that emergency use of such product is authorized under section 564 of the Federal Food, Drug and Cosmetic Act.
>
> Provision of Information. If the President, under subsection (a) waives the condition described in section 564(e)(1)(A)(ii)(III) of the Federal Food, Drug and Cosmetic Act, and if the Secretary of Defense, in consultation with the Secretary of Health and Human Services, makes a determination that it is not feasible based on time limitations for the information described in section 564(e)(1)(A)(ii)(I) or (II) of such Act and required under paragraph (1)(A) or (2)(A) of such section 564(e), to be provided to a member of the armed forces (or next-of-kin in case of the death of a member) to whom the product was administered as soon as possible, but not later than 30 days after such administration. The authority provided for in this subsection may not be delegated. Information concerning the administration of the product shall be recorded in the medical record of the member.
>
> Applicability of Other Provisions. In the case of an authorization by the Secretary of Health and Human Services under section 564(a)(1) of the Federal Food, Drug, and Cosmetic Act based on a determination of the Secretary of Defense under section 564(b)(1)(B) if such Act, subsections (a) through (f) of section 1107 shall not apply to the use of a product that is the subject of such authorization within the scope of such authorization and while such authorization is effective.[63,64]

The Turner Bill

Another bill (HR 4258, Rapid Pathogen Identification to Delivery of Cures Act), introduced by Congressman Jim Turner et alia on May 4, 2004, allows research and development of medical countermeasures and diagnostics to move at a quicker pace so that new products can rapidly be made available for emergencies. In addition, the Turner Bill provides for research and development of drugs and vaccines against genetically modified pathogens not accounted for in the Project BioShield legislation, which covered only countermeasures related to existing unmodified threat agents.

Project BioShield and the Turner Bill together establish an FDA EUA for critical biomedical countermeasures. The FDA may approve solely for emergency use a product not approved for full commercial marketing. For products that are near final approval but may not have met all the criteria, the FDA has created a streamlined IND process, with the animal efficacy rule playing a central role, for products designed to protect against or treat conditions caused by nuclear, chemical, or biological terrorism. Such a process was used to obtain FDA approval for pyridostigmine, which is licensed for use in treating myasthenia gravis and was approved on February 5, 2003, for use to increase survival after exposure to soman nerve gas poisoning by the military in combat.[65]

Biodefense and Pandemic Vaccine and Drug Development Act Renewal of 2011

In October 2005, Senator Richard Burr of North Carolina introduced the Biodefense and Pandemic Vaccine and Drug Development Act of 2005 (S 1873). This bill establishes the Biomedical Advanced Research and Development Agency as the lead federal agency for the development of countermeasures against bioterrorism. The new agency would report directly to the secretary of Health and Human Services. The bill provides incentives for domestic manufacturing of vaccines and countermeasures, and it gives broad liability protections to companies that develop vaccines for biological weapons. This bill may appear to settle the residual concerns left unresolved by Project BioShield, but it has raised additional controversy because of public perceptions that it is too favorable to the pharmaceutical industry and issues related to secrecy provisions.

In March 2011, the Pandemic and All-Hazards Preparedness Act Reauthorization Act of 2011 received US Senate approval.[66] The act renewed measures from the 2006 legislation to promote the development and procurement of medical countermeasures against weapons of mass destruction agents. It reauthorized Project Bioshield Special Reserve Fund for purchasing vaccines and other treatments over a 10-year period. Funding for biodefense has now evolved to include nonbiodefense and emerging pathogens research, development, and product acquisition with continued government financial increases since the original act in 2005.[67]

DILEMMAS FOR BIODEFENSE RESEARCH

The potential devastating consequences of a bioterrorist event was revealed to the public in 2001, and this fear was enhanced by its proximity in time to the tragic events of 9/11. The immediate reaction of the government and the public was to support new legislation intended to protect the homeland (Patriot Act) and expand the law enforcement, military, intelligence, and defense industries. Funding was dramatically

increased to agencies funding biodefense research and development. Tens of billions of dollars were spent on building infrastructure, establishing personal reliability/biosurety/biosecurity oversight, expanding the number of biocontainment laboratories, hiring containment specialists, training first responders, stockpiling personal protective equipment, establishing laboratory response networks and biodetection capabilities, establishing stockpiles of medical countermeasures, and greatly expanding biodefense research and development.[68]

Dual use research consists of scientific studies that possess the potential of generating information or material that—if used nefariously—could harm public health.[69–75] This dichotomy often results in the placement of potentially excessive restrictions and limitations upon the types of research permitted, and thus has the potential of preventing or severely restricting the ability of scientists to find solutions or answer key questions needed to mitigate biothreats. For example, performing genetic modifications to produce antibiotic resistant bacterial select agents or creating novel recombinant strains of influenza are prevented under the Biological Weapons and Toxins Convention and the United States Government Policy for Oversight of the Life Sciences Dual Use Research. Without access to these modified organisms, determining the efficacy of potential new therapies or vaccines using in vitro assays and in vivo animal models is hampered, creating fundamental gaps in our biodefensive posture and national security. A critical need exists for a review of ethics in biodefense[76] and the researchers responsible to safely and securely find solutions to the biodefense related problems.[77]

SUMMARY

This chapter has provided a view of the history of ethically conducted human subject research in the military and has presented some of the problems that still exist among the distinct regulatory bodies that impact this research. The DoD has an ethical responsibility to protect military personnel, yet there is disagreement over how to best protect them against biological warfare attacks, in light of equal commitments to respecting agency autonomy and limiting government power over individual decisions regarding what constitutes one's own best interests. These issues and problems are not a mystery to those who confront them on a daily basis, and many thoughtful individuals are focusing their attention on resolving these dilemmas. Some progress is being made, at least in terms of productive dialogue and substantive attention to legislation that might impact research.

Acknowledgments

The authors would like to acknowledge the efforts of the following individuals in preparing this chapter. Lee L Zwanziger, PhD, while Director of Research on the Staff of the President's Council on Bioethics, provided valuable advice and criticism during the early phases of writing. Jonathan D Moreno, PhD; Emily Davie; Joseph S Kornfeld, Professor of Biomedical Ethics at the University of Virginia and Director, Center for Biomedical Ethics; and Griffin Trotter, PhD, MD, Associate Professor in the Center for Health Care Ethics at Saint Louis University provided critical reviewers' comments.

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ABBREVIATIONS AND ACRONYMS

A

AAALAC: Association for Assessment and Accreditation of Laboratory Animal Care
ABC: ATP-binding cassette
ABSA: American Biological Safety Association
ABSL: animal biosafety level
ACB: *Acinetobacter calcoaceticus baumannii*
ACEI: angiotensin-converting enzyme inhibitor
ACIP: Advisory Committee on Immunization Practices
ADAMs: A Disintegrin-Like And Metalloproteinase-containing protein
Ads: adenoviruses
Ag-ELISA: antigen enzyme-linked immunosorbent assay
agr: accessory gene regulator
AHF: Argentinian hemorrhagic fever
AI: avian influenza
AIDS: acquired immune deficiency syndrome; acquired immunodeficiency syndrome
AIGIV: anthrax immune globulin intravenous (human)
ALO: anthrolysin O
α-DG: α-dystroglycan
AML: Area Medical Laboratory
AMR: antimicrobial resistance monitoring
ANG: Air National Guard
Ank: ankyrin repeat domain
AnkG: ankyrin repeat-containing protein G
AP-1: activator protein 1
APC: antigen-presenting cell
APHIS: Animal and Plant Health Inspection Services
APSV: Aventis Pasteur smallpox vaccine
AR: Army Regulation
ARDS: acute respiratory distress syndrome
ARM: antimicrobial resistance monitoring
ARMoR: Antimicrobial Resistance Monitoring and Research (Program)
ARO: alternate responsible official
ASD(HA): Assistant Secretary of Defense for Health Affairs
ASP: amnesic shellfish poisoning
AT: autotransporter
ATLS: Advanced Trauma Life Support
ATP: adenosine 5′-triphosphate
AVA: anthrax vaccine adsorbed
AVP: anthrax vaccine precipitated

B

BAMC: Brooke Army Medical Center
BAT: botulism antitoxin heptavalent
BBB: blood–brain barrier
BDBV: Bundibugyo virus
BEBOV: Bundibugyo ebolavirus
BHF: Bolivian hemorrhagic fever
BIG: Botulism Immune Globulin Intravenous
BMBL: *Biosafety in Microbiological and Biomedical Laboratories*
BoNT: botulinum neurotoxin
BPRP: Biological Personnel Reliability Program
BSAT: biological select agents and toxins
BSC: biological safety cabinet
BSL: biosafety level
BSP: Biological Surety Program
BT: bioterrorism
BTWC: Biological and Toxin Weapons Convention
BuHA: *Burkholderia* Hep_Hag autotransporter
BW: biological warfare
BWC: Biological Weapons Convention

C

C: capsid
cAd3-EBOZ: chimpanzee adenovirus type 3–vectored ebolavirus Zaire vaccine
CAdVax: complex adenovirus vaccine
CAFO: concentrated animal feeding operation
CAP: College of American Pathologists
CARA MEL: CBRNE Analytical & Remediation Activity Mobile Expeditionary Laboratory
CBDP: Chemical Biological Defense Program
CBIRF: Chemical-Biological Incident Response Force
CBRN: chemical, biological, radiological, and nuclear
CBRNE: chemical, biological, radiological, nuclear, and explosives
CBSP: Certified Biological Safety Professional
CC_{50}: modeling of cytotoxicity data
CCD: colony collapse disorder
CCHFV: Crimean–Congo hemorrhagic fever virus
CCP: critical control point
CD: Cluster of Differentiation
CD-22: Camp Detrick-22
CDC: Centers for Disease Control and Prevention
CDC-DSAT: Centers for Disease Control and Prevention-Division of Select Agents and Toxins
CEV: cell-associated enveloped virion
CFR: case fatality rate; Code of Federal Regulations
CFT: cell-free translation; complement fixation test
CFU/cfu: colony-forming unit
CHAPV: Chapare virus
CHIK: chikungunya
CHIKV: chikungunya virus
CHOC: chocolate agar
CLDC: cationic liposome DNA complex
CLIA: Clinical Laboratory Improvement Amendments
CLIP: Clinical Laboratory Improvement Program
CLSI: Clinical Laboratory Standards Institute
CMA: competent medical authority
CMI: cell-mediated immune
CNS: central nervous system
CO: certifying official
CONUS: continental United States
CoV: Coronavirus
CP: capsid protein
CpG: cytosine-phosphate-guanine
CPS: capsular polysaccharide
CSF: cerebrospinal fluid
CSH: Combat Support Hospital
CST: Civil Support Teams
CT: computed tomography
CWC: Chemical Weapons Convention

D

DA: US Department of the Army
DA PAM: Department of the Army Pamphlet
dB: decibel
DEET: *N,N*-diethylmetatoluamide
DENV: dengue virus
DHF: dengue hemorrhagic fever
DHHS: Department of Health and Human Services
DHS: Department of Homeland Security
DIC: disseminated intravascular coagulation
DNA: deoxyribonucleic acid
DoD: Department of Defense
DoD-GEIS: Department of Defense-Global Emerging Infections Surveillance and Response System

DoDI: Department of Defense Instruction
DON: deoxynivalenol
dot/icm: defective in organelle trafficking/intracellular multiplication (lc)
DPP4: dipeptidyl peptidase 4
DRC: Democratic Republic of the Congo
DSAT: Division of Select Agent and Toxins
DSS: dengue shock syndrome
DURC: dual use research of concern

E

EAEC: enteroaggregative *Escherichia coli*
EBOV: Ebola virus
EC_{50}: concentration of a drug that gives half-maximal response
ECL: electrochemiluminescence
EDTA: ethylenediaminetetraacetic acid
EEE: eastern equine encephalitis
EEEV: Eastern equine encephalitis virus
EEV: extracellular enveloped virion
EIEC: enteroinvasive *Escherichia coli*
EHEC: enterohemorrhagic *Escherichia coli*
ELISA: enzyme-linked immunosorbent assay
EMDG: Expeditionary Medical Dental Group
EPA: US Environmental Protection Agency
EPEC: enteropathogenic *Escherichia coli*
EPICON: epidemiological consultation
EPS: exopolysaccharide
ER: endoplasmic reticulum
Erks: extracellular signal-regulated kinases
ESBL: extended-spectrum β-lactamase
ESF: emergency support function
ESKAPE: *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter calcoaceticus baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter*
ET: edema toxin
EUA: Emergency Use Authorization
EV: enveloped virion
EVD: Ebola virus disease

F

F: fusion protein
F1: fraction 1
Fab: fragment antigen-binding
FBI: Federal Bureau of Investigation
FDA: Food and Drug Administration
FEMA: Federal Emergency Management Agency
FFAG: flow-focusing aerosol generator
FFBAT: Air Force Biological Augmentation Team
FHP: force health protection
fla B: flagellin B gene
FMD: foot and mouth disease
FPI: *Francisella* pathogenicity island
FSAP: Federal Select Agent Program

G

G: attachment glycoprotein
Gal/GalNac: *N*-acetyl galactosamine
GCP: Good Clinical Practices
G-CSF: granulocyte colony-stimulating factor
GEIS: Global Emerging Infections Surveillance and Response System
GMP: good manufacturing practices
GPC: glycoprotein precursor
GTOV: Guanarito virus
GTX: gonyautoxin

H

HA: hemagglutinin
HACCP: hazard analysis critical control point
HAI: healthcare-associated infection/ hospital-associated infection
HAVCR1: hepatitis A virus cellular receptor 1
HAZMAT: hazardous materials
HBAT: heptavalent botulinum antitoxin
HBsAg: hepatitis B surface antigen
Hc: heavy chain
HCoV: human Coronavirus
HDP: 1-0-hexadecyl-oxypro-pyl
HEPA: high-efficiency particulate air
HeV: Hendra virus
HFRS: hemorrhagic fever with renal syndrome
HHA: hand held assay
HHS: US Department of Health and Human Services
HI: hemagglutination inhibition
HIV: human immunodeficiency virus
HLA: Human Leukocyte Antigen
HPAI: highly pathogenic avian influenza
HPV: human papilloma virus
HTS: high throughput screening
HUS: hemolytic uremic syndrome

I

IBC: Institutional Biosafety Committee
IC_{50}: half-maximal inhibitory concentration
ICAM-1: intercellular adhesion molecule-1
ICC: International Clonal Complex
ICD-10: *International Statistical Classification of Diseases and Related Health Problems, Tenth Revision*
ICLN: Integrated Consortium of Laboratory Networks
ICS: Incident Command System
IEV: intracellular enveloped virion
IFA: indirect immunofluorescence assay
IFN: interferon
IFNγ: interferon gamma
IgG: immunoglobulin G
IgM: immunoglobulin M
IHA: direct hemagglutination assay
IHR: International Health Regulations
IL: interleukin
IM: intramuscular/intramuscularly
IMP: inosine 5′-phosphate
IMV: intracellular mature virion
IN: intranasal
IND: investigational new drug
IP: intraperitoneal
IQ: ilimaquinone
IRB: institutional review board
IT: intratracheal
IQ: ilimaquinone
IU: international unit
IV: intravenous

J

JBAIDS: Joint Biological Agent Identification and Diagnostic System
JEV: Japanese encephalitis virus
JUNV: Junín virus

K

KPC: *Klebsiella pneumoniae* carbapenemase

L

L: large; large RNA-dependent RNA polymerase
LAAV: live attenuated anthrax vaccine
LAMP: lysosomal-associated membrane protein
LASV: Lassa virus
LC/MS: liquid chromatography/mass spectrometry
LCt_{50}: acute toxicity
LD_{50}: lethal dose (amount necessary to kill 50% of the subject population)
LFA-1: lymphocyte function-associated antigen
LLAP: Legionella-like amoebal pathogen
LLOV: Lloviu virus
LOD: limit of detection
LPC: lysophosphatidylcholine
LPCR: large-fragment polymerase chain reaction
LPS: lipopolysaccharide
LRMC: Landstuhl Regional Medical Center
LRN: Laboratory Response Network
LRN-B: Laboratory Response Network-Biological
LSD: lysergic acid diethylamide
LT: lethal toxin; long term
LUJV: Lujo virus
LVS: live vaccine strain

M

M: matrix protein
mAb/mAbs: monoclonal antibody/monoclonal antibodies
MACV: Machupo virus
MADV: Madariaga virus
MALDI-TOF MS: matrix-assisted laser desorption/ionization-time of flight mass spectrometry
MAP: mitogen-activated protein
MAPK: mitogen-activated protein kinase
MARV: Marburg virus
MASCAL: mass casualty event or exercise
Mb: megabase pair
MCM: medical countermeasure
MCP-1: monocyte chemoattractant protein-1
MDCK: Madin-Darby Canine Kidney cells
MDR: multidrug resistant
MDRO: multidrug-resistant organism
MEDCOM: US Army Medical Command
MEDEVAC: medical evacuation
MERS: Middle East Respiratory Syndrome
MH: Madagascar hissing
MHC: major histocompatibility complex
MHS: Military Health System
MIC: mean inhibitory concentration
miRNA: microRNA
MLST: multilocus sequence typing
MLVA: multiple-locus variable number tandem repeat analysis
MMAD: mass median aerodynamic diameter
MNGC: multinucleated giant cell
MOU: memorandum of understanding
MPL: 3-*O*-desacyl-4′-monophosphoryl lipid A
MRSN: Multidrug-Resistant Organism Repository and Surveillance Network
MS: mass spectroscopy
MSD: Meso Scale Discovery
MTF: medical treatment facility
mTOR: mammalian target of rapamycin
MVA: Modified Vaccinia Ankara/modified vaccinia virus Ankara
MVD: Marburg virus disease
MW: molecular weight

N

N: nucleocapsid protein
NA: neuraminidase; North American
NAS: National Academy of Sciences
NATO: North Atlantic Treaty Organization
NDM: New Delhi metallo-β-lactamase-1
NDMS: National Disaster Medical System
NDRF: National Disaster Recovery Framework
NF-AT: nuclear factor of activated T cells
NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
NGDS: Next Generation Diagnostic System
NGS: next-generation sequencing
NHP: nonhuman primate
NIAID: National Institute of Allergy and Infectious Diseases
NIH: National Institutes of Health
NIOSH: National Institute of Occupational Safety and Health
NiV: Nipah virus
NIV: nivalenol
NLR: NOD-like receptor
NMDA: *N*-methyl-D-aspartate
NNMC: National Naval Medical Center
NOD: nucleotide oligomerization domain
NP: nucleoprotein
NPG: National Preparedness Goal
NRF: National Response Framework
NSABB: National Science Advisory Board for Biodefense
NSP: neurotoxic shellfish poisoning

O

OCONUS: outside of the continental United States
OEF: Operation Enduring Freedom
OFPBL: Oxidative-Fermentative-Polymyxin B-Bacitracin-Lactose (agar)
OIF: Operation Iraqi Freedom
OPS: *O*-polysaccharide
OR: operating room
OSHA: Occupational Safety and Health Administration
o/w: oil in water

P

P: phosphoprotein
PA: protective antigen
pAbs: polyclonal antibodies
PAPR: powered air-purifying respirator
PBT: pentavalent botulinum toxoid
PCR: polymerase chain reaction
PDI: potentially disqualifying information
PEG: polyethylene glycol
PEP: postexposure prophylaxis
PFGE: pulsed field gel electrophoresis
PHV: parvovirus-like hybrid virus
PI: principal investigator
PI3K: phosphoinositide 3 kinase
PKC: protein kinase C
PLA2: phospholipase A2
Poly I:C: polyinosinic:polycytidylic acid
PPE: personal protective equipment
PPES: positive-pressure encapsulating suit
PRNT: plaque reduction neutralization test
$PRNT_{80}$: 80% plaque reduction neutralization titer
PS: polysaccharide
PSI: personnel security investigation
PSP: paralytic shellfish poisoning; phage shock protein
PTA: pteroic acid
PTK: protein tyrosine kinase
PV: parasitophorous vacuoles

Q

QS: quorum sensing
Q-Vax: Q fever vaccine

R

rAb: recombinant antibody
RAMP: Rapid Analyte Measurement Platform
RANTES: regulated upon activation normal T-cell expressed, and secreted
RAVV: Ravn virus
rBimA: recombinant *Burkholderia* intracellular motility A
RBP: Registered Biosafety Professional
rDNA: ribosomal deoxyribonucleic acid
RDTE: research, development, test, and evaluation
RESTV: Reston virus
RFLP: restriction fragment-length polymorphism
RIFLE: risk, injury, failure, loss, and end-stage renal disease
RIP: ribosome inactivating protein
RNA: ribonucleic acid
RNP: ribonucleoprotein
RO: responsible official
RR: relative risk
RSV: respiratory syncytial virus
RTA: ricin A chain
RTB: lectin B-chain
RT-PCR: reverse transcription/transcriptase polymerase chain reaction
RVFV: Rift Valley fever virus

S

S: small
SA: South American
SABV: Sabiá virus
SARS: severe acute respiratory syndrome
SARS-CoV: severe acute respiratory syndrome-Coronavirus
SAT: select agents and toxins
SBA: sheep blood agar
SD: standard deviation
SDA: Seventh-day Adventist
SdAbs: single domain antibodies
SE: staphylococcal enterotoxin
SEA: staphylococcal enterotoxin A
SEB: staphylococcal enterotoxin B
SEBOV: Sudan ebolavirus
SFTS: severe fever with thrombocytopenia syndrome
SFTSV: severe fever with thrombocytopenia syndrome virus
SFV: Semliki Forest virus
sGP: secreted glycoprotein
SINV: Sindbis virus
SIP: Special Immunizations Program
SMX: sulfamethoxazole
SNARE: soluble *N*-ethylmaleimide-sensitive factor activating protein receptor
SNS: Strategic National Stockpile
SOP: standard operating procedure
SPE: streptococcal pyrogenic exotoxin
SRA: security risk assessment
ssGP: secondary secreted glycoprotein
ST: sequence type
STAG: spinning top aerosol generator
STEC: Shiga toxin-producing *Escherichia coli*
STRIVE: Sierra Leone Trial to Introduce a Vaccine Against Ebola
STX: saxitoxin
SUDV: Sudan virus

T

T3SS: type three secretion system
T6SS: type VI secretion system
TAFV: Taï Forest virus
Tat: twin arginine transport
TBEV: tickborne encephalitis virus
$TCID_{50}$: median tissue culture infective/infecting dose
TCR: T-cell receptor
3D: three-dimensional
TIR: toll/IL-1 receptor
TLR: toll-like receptor
TMP: trimethoprim
TMP-SMZ: trimethoprim combined with sulfamethoxazole
TNF-α/TNFα: tumor necrosis factor-alpha
TrD: Trinidad donkey
TSS: toxic shock syndrome
TSST-1: toxic shock syndrome toxin-1
TTSS: type III secretion system

U

UK: United Kingdom
UN: United Nations
UNSCOM: UN Special Commission
USAMRIID: US Army Medical Research Institute of Infectious Diseases
USAMU: US Army Medical Unit
USA PATRIOT Act: Uniting and Strengthening America by Providing Appropriate Tools Required to Intercept and Obstruct Terrorism Act of 2001
USC: United States Code
USDA: US Department of Agriculture
USNS: United States Naval Ship
USSR: Union of Soviet Socialist Republics (or the Soviet Union, now Russia)

V

V: variable
VACV: vaccinia virus
VAERS: Vaccine Adverse Event Reporting System
VEE: Venezuelan equine encephalitis
VEEV: Venezuelan equine encephalitis virus
VHF: Venezuelan hemorrhagic fever; viral hemorrhagic fever
VIG: vaccinia immune globulin
VLP: virus-like particle
VLS: vascular leak syndrome
Vm: minute volume
VRP: virus-like replicon particle
VS: virtual screening
VSV: vesicular stomatitis virus
vWF: von Willebrand factor

W

WCV: whole cell vaccine
WEE: Western equine encephalitis
WEEV: Western equine encephalitis virus
WHO: World Health Organization
WMD: weapons of mass destruction
WNV: West Nile virus
w/o: water in oil
WRAIR: Walter Reed Army Institute of Research
WRAMC: Walter Reed Army Medical Center
WS: working stock
WWI: World War I
WWII: World War II

Y

Yaps: *Yersinia* autotransporter proteins
YFV: Yellow fever virus

Z

Z: zinc-binding matrix protein
ZEBOV: Zaire ebolavirus
ZF: zinc-finger

INDEX

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