
MEDICAL ASPECTS OF BIOLOGICAL WARFARE



The Coat of Arms
1818
Medical Department of the Army

A 1976 etching by Vassil Ekimov of an original color print that appeared in *The Military Surgeon*, Vol XLI, No 2, 1917

The first line of medical defense in wartime is the combat medic. Although in ancient times medics carried the caduceus into battle to signify the neutral, humanitarian nature of their tasks, they have never been immune to the perils of war. They have made the highest sacrifices to save the lives of others, and their dedication to the wounded soldier is the foundation of military medical care.

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On October 12, 2007, during a planned exercise conducted by the Aeromedical Isolation Team of the US Army Medical Research Institute of Infectious Diseases at Fort Detrick, Maryland, a patient who has notionally been exposed to a biological agent is being contained in the stretcher transit isolator and being prepared for transport via helicopter to be given medical care in the biosafety level-4 containment care suite (“the slammer”).

Photograph by Bruce Maston, 2007.

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Foreword

Our world was dramatically altered by the terrorist attacks of September 11, 2001. This assault, the yet unsolved mailings of anthrax, and other threats oblige a renewed national attention to the threat of biological weapons. The term “warfare” is no longer limited to conventional battlefields. Now we are concerned about the more likely scenario—wanton acts of biological terrorism inflicted on unsuspecting citizens anywhere in the world.

We must counter this threat with vigilance and maximize our response to attack with our best medical practices to identify agents involved, minimize casualties, and expedite the treatment of survivors. Our Nation charges the Armed Forces to guard against bioattack—overt or covert—as well as managing recovery efforts. This new groundbreaking volume in the Textbooks of Military Medicine series, devoted to biological warfare and terrorism, responds to that charge.

Since the publication of *Medical Aspects of Chemical and Biological Warfare* more than a decade ago, the editors at Borden Institute and the respective medical leaders across the Army Medical Command concluded that this essential new information required stand-alone textbooks. This affords the specific medical hazards a more detailed assessment and attention. I believe they succeeded in that effort.

Grounded in a historical perspective, this new volume, *Medical Aspects of Biological Warfare*, addresses weaponization of biological agents. It categorizes potential agents as food, waterborne, or agricultural toxins and discusses the respective epidemiology. A description of individual agents includes recent advances in the knowledge base and the illnesses induced. The authors present familiar (anthrax, plague, smallpox) and less often discussed biotoxins (alphaviruses, staphylococcal enterotoxins) and explain methods for early agent identification. To maximize understanding, authors used case studies and research along with successful management practices, treatments, and antidotes.

The description of the practical issues related to civil defense and the inherent differences between national, state, and metropolitan priorities with regard to biosurety, quarantine, crisis management, public affairs, and legal considerations is clear. The potential dangers of emerging infectious diseases and their threat to public safety did not interfere with clear presentation of “here-and-now” risks. The editors conscientiously present the ethical aspects of preparing for scenarios that by their nature are unknowable, unethical, or unforeseen.

The publication of this volume establishes best practices in the field of biohazard management, thus making those best practices available to healthcare practitioners, policy makers, and planners, in and out of uniform. Some will challenge our release of a textbook on the topic of bioweapons—they claim it is wiser, safer, and more prudent to withhold this information in the interest of better safeguarding our citizens. We maintain that in any analysis, the strongest safeguard of a free society is the open forum and free exchange of science, ideas, and theory. Regardless of your perspective, this text is excellent and I am extremely proud of the professionals who devoted their time and talents to it.

Major General Gale S. Pollock
Acting, The Surgeon General
US Army

Washington, DC
November 2007

Preface

Medical defense against biological pathogens used in terrorism or warfare has emerged over the past decade from the workings of a few select research laboratories to an expansive undertaking by the federal government. Largely the domain of military medical defense facilities, events post-2001 have led to tremendous investments in infrastructure, public health response, and basic research to medically defend against these identified threats. The Department of Defense efforts have been eclipsed to a degree by the scope of investments by the Department of Health and Human Services and the Department of Homeland Security. One area, however, that remains critical is the need to transfer the resulting information and best medical practices to the medical practitioners. The Department of the Army has maintained a leadership role in this crucial enterprise.

The history of biological weapons use by nations and terrorist groups necessitates a high level of preparedness for uniformed healthcare providers and scientists. Much of what is understood as standards of practice served the United States well during the events related to the 2001 anthrax mailings, yet important lessons were learned from that unique experience. The continued threat of biological weapons dictates that all Department of Defense medical personnel become conversant with state-of-the art treatment for biological casualties. What may have been perceived merely as useful information in the past is now a requirement for medical providers.

The previous edition of *Medical Aspects of Chemical and Biological Warfare* in the Borden Institute's Textbooks of Military Medicine series was both innovative and much needed at the time of publication in 1997. In his foreword, then Army Surgeon General Ronald Blanck stated that "world events have conspired to increase the threat of use of chemical and biological weapons." A decade later, the complexity of the threat has increased beyond the boundaries of state-sponsored programs and to the terrorist use of novel pathogens. The need for a revised version of this work has never been greater. It is with great pride that I introduce the reader to the new edition of *Medical Aspects of Biological Warfare*. The scientists and physicians who are responsible for this text have endeavored to provide the best possible biomedical reference.

Colonel George W. Korch
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Fort Detrick, Maryland
July 2007

The current medical system to support the US Army at war is a continuum from the forward line of troops through the continental United States; it serves as a primary source of trained replacements during the early stages of a major conflict. The system is designed to optimize the return to duty of the maximum number of trained combat soldiers at the lowest possible level. Far-forward stabilization helps to maintain the physiology of injured soldiers who are unlikely to return to duty and allows for their rapid evacuation from the battlefield without needless sacrifice of life or function.

Chapter 1

HISTORY OF BIOLOGICAL WEAPONS: FROM POISONED DARTS TO INTENTIONAL EPIDEMICS

JAMES W. MARTIN, MD, FACP^{*}; GEORGE W. CHRISTOPHER, MD, FACP[†]; AND EDWARD M. EITZEN, JR, MD, MPH[‡]

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SUMMARY

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INTRODUCTION

Since prehistoric times, humans have used available technologies for destructive and beneficial purposes. 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, *τοξικον 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 weapons 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. However, 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, terrorist organizations, and criminal elements is likely to continue.

EARLY ATTEMPTS

The early use of biological weapons included the contamination of water with animal carcasses and filth. Another ancient tactic was to allow an enemy to take sanctuary in an area endemic for an infectious agent in anticipation that the enemy force would become infected, for example, allowing unimpeded access of opposing forces to areas where transmission of malaria was highly likely.

The Carthaginian leader, Hannibal, used early biological weapons (serpent toxins) in the naval battle of the Eurymedon against King Eumenes of Pergamum in 184 BCE. Hannibal ordered earthen pots filled with serpents to be hurled onto the decks of the Pergamene ships. The pots shattered on impact, releasing live serpents among the enemy sailors. The Carthaginians exploited the ensuing panic and chaos to win the battle.³

One of the most notorious early biological warfare methods was the hurling of cadavers over the walls of besieged cities, primarily as a terror tactic. De Mussis provided a dramatic record of the use of plague victims in biological warfare.^{4,5} After war broke out between the Genoese and the Mongols in 1343 for control of the lucrative caravan trade route from the Black Sea to the Orient, the Mongols laid siege to Caffa, a Genoese colony in the Crimea. 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 lift 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 of the incident by Wheelis⁵ suggests that the introduction of plague into the city by the cadavers—as a result of a tactically successful biological attack—is the most biologically plausible of several competing hypotheses on the source of the

outbreak. Although historically the predominant mode of human plague transmission has been attributed to bites from infected fleas, modern experience (United States 1970–1995)⁶ has implicated direct transmission from contact with infected (animal) carcasses in 20% of instances in which the source of the infection could be attributed epidemiologically. Contact with tissue and blood would have been inevitable during the disposal of hundreds or possibly thousands of cadavers that had been smashed on impact. Typically, 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 front line and the city walls.⁵ Transmission from sylvatic to urban rodents is infrequent, at least under current ecological conditions.⁷ Alternatively, plague could have been introduced by imported human cases or infected rodents brought into the city through the maritime trade, which was maintained during the siege. Regardless of the portal of entry, the epidemic was likely amplified by an increase in the population of rats and fleas under siege conditions.

Smallpox was particularly devastating to Native Americans. The unintentional yet catastrophic introduction of smallpox to the Aztec empire during the Narváez expedition of 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.⁸ At the conclusion of the French and Indian War in 1763, the Native Americans conducted a series of attacks against British forts along the western frontier in what is known as Pontiac’s Rebellion. An outbreak of smallpox at Fort Pitt presented an opportunity to take advantage of the Native Americans’ unique susceptibility to this disease.⁹ On May 24, 1763, William Trent, the local militia leader, wrote of the actions of Captain Ecuyer, the Fort Pitt commander: “We gave them two Blankets and a Handkerchief from

the Smallpox Hospital. I hope it will have the desired effect."^{10,11} Subsequently (in July 1763), Sir Jeffrey Amherst, British commander of forces in the American colonies, conceptualized a similar plan with Colonel Henry Bouquet, apparently unaware of the actions at Fort Pitt, thus sanctioning the concept of use of smallpox as a biological weapon.^{12,13} An epidemic of smallpox occurred among the Native Americans of the Ohio River Valley that year. In retrospect, it is difficult

to evaluate the tactical success of Captain Ecuyer's biological attack because smallpox may have been transmitted after other contacts with colonists, as had previously happened in New England and the South. Although scabs from smallpox patients are thought to be of low infectivity as a result of binding of the virus in fibrin matrix, and transmission by fomites has been considered inefficient compared with respiratory droplet transmission.⁸

THE EARLY ERA OF MODERN MICROBIOLOGY AND 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 the possible use of bacteriological weapons was not specifically identified or addressed.^{14,15} Germany started the first known scientific, state-sponsored biological weapons program during World War I.¹⁶ German espionage agents reportedly undertook a covert biological campaign in the United States before the United States entered the war. The Allies had purchased US draft animals for military use, and German operatives infected these animals with glanders and anthrax while they were awaiting shipment overseas.¹⁷ The Germans also conducted similar operations in Romania, Russia, Norway, Mesopotamia, and Argentina, with varying levels of success. Attempts were also made to cripple grain production in Spain with wheat fungus, but without success.¹⁸

The German biowarfare program of World War I is of special interest for several reasons: it was the first national offensive program, the first program to have a scientific foundation, and the first confirmed instance of actual wartime use of biological agents. The German program was a large-scale (strategic) biological attack, which targeted neutrals rather than belligerents and targeted crops and animals as opposed to humans. It is impossible to determine the effectiveness of this program; although the German operatives involved thought it was a success, no documentary evidence supports this conclusion.¹²

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. The protocol, developed by the League of Nations' Conference for the Supervision of the International Trade in Arms and Ammunition, addressed warfare methods of nation-states only. 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 basic 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.¹⁹

After the Japanese defeat of Russia in the 1905 Russo-Japanese War, Japan became the dominant foreign power in Manchuria. The Kwantung Army was created to maintain Japanese economic interests in the region. During the 15 months from September 1931 to the end of 1932, the Japanese military seized full control of Manchuria. In 1932 Major Shiro Ishii, a Japanese army physician with an established interest in biological agents, came to Harbin to conduct human research. He established his initial laboratory in the industrial sector of Harbin known as the Nangang District, 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 experimenting on a more dramatic scale. No research study subjects survived; all died of either experimental infection or live vivisection. These studies continued until a prisoner riot and escape occurred, which resulted in the closing of the facility in 1937. However, larger and more extensive facilities were subsequently built.¹⁹

In August 1936 Ishii, now promoted to Lieutenant Colonel, was made chief of the Kwantung Army water purification bureau. That autumn the Japanese appropriated 6 km² of farmland 24 km south of Harbin, which encompassed 10 villages and displaced 600 families from their ancestral homes. There Ishii built the massive research facility known as Unit 731, where a census of 200 prisoners was kept as expendable subjects of experimentation. Ultimately, more than 3,000 Chinese prisoners were killed and cremated after these experiments. Most of the evidence was destroyed at the end of the war, and in all likelihood the actual number was much greater.¹⁹

Major Wakamatsu Yujiro, a less flamboyant but equally ruthless veterinary officer, ran the Unit 100

facility at Changchun. In 1936 Japan appropriated 20 km² of land near Mokotan, a small village just 6 km south of Changchun, the capital of Japanese-occupied Manchuria. Predominantly a veterinary and agricultural biowarfare research unit (independent from Ishii's Unit 731), Unit 100 focused on developing biological weapons for sabotage operations. Although animals and crops were the focus of most of the research, numerous human studies were also conducted, similar to those conducted by Unit 731.¹⁹

In April 1939 a third major research facility, Unit Ei 1644, was established in an existing Chinese hospital in Nanking, under the command of one of Ishii's lieutenants, Lieutenant Colonel Masuda. Prisoners, including women and children, became the subjects of grisly experimentation, and were cremated in the camp incinerator usually late at night. Chemical warfare experiments were conducted in a gas chamber with an observation window. Unit Ei 1644 supported Unit 731's research efforts with bacterial agent production and flea cultivation.¹⁹

Eleven Chinese cities were allegedly attacked during "field trials" using infectious agents including *Yersinia pestis*, *Vibrio cholerae*, and *Shigella*. These attacks may have backfired because up to 10,000 Japanese soldiers reportedly contracted cholera after a biological attack on Changde in 1941.²⁰ As a result of the Japanese biowarfare program, 580,000 people are estimated to have died in China. The field trials were terminated in 1943, yet basic research and human experimentation at Unit 731 and elsewhere continued until the end of the war.^{19,21}

Vaccine research and development was conducted at both Tokyo University and Unit 731. By the end of the war, the Japanese biowarfare program claimed to have effective vaccines for anthrax, cholera, dysentery, typhoid, and typhus. Unit 731 reportedly produced 20 million doses of vaccine per year, with millions more doses produced at satellite facilities in Manchuria and other parts of China. Use of biological warfare agents by Japanese forces may have given them an advantage over the Chinese, but results were erratic and prone to backfire. 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.¹²

In contrast to Japanese efforts during World War II, a German offensive biological weapons program never materialized. Studies of experimental infections using prisoners were done primarily to study pathogenesis and develop vaccines and sulfonamide antibiotics, rather than to develop biological weapons. Hitler re-

portedly issued orders prohibiting biological weapons development. With the support of high-ranking Nazi party officials, however, German scientists began biological weapons research, but their results lagged far behind those of other countries.²²

Polish physicians used a vaccine and a serologic test during World War II in a brilliant example of "biological defense." Knowing that inoculation with killed *Proteus* OX-19 would cause a false-positive Weil-Felix typhus test, Polish physicians inoculated the local population with a preparation of formalin-killed *Proteus* OX-19 to create a serologic pseudoepidemic of typhus. Using serologic surveillance, the German army avoided areas that appeared to contain epidemic typhus; consequently, residents of these areas were spared deportation to concentration camps.²³ Several reported but unconfirmed allegations indicate that Polish resistance fighters conducted biological warfare against Nazi occupation forces, including using letters contaminated with *Bacillus anthracis* to cause cases of cutaneous anthrax among Gestapo officials^{18,24} and using typhus against German soldiers.^{18,25} Czechoslovakian agents reportedly used a grenade contaminated with botulinum toxin, supplied by British Special Operations, to assassinate Reinhard Heydrich, the Nazi governor of occupied Czechoslovakia²⁶; however, the veracity of this reported incident has been challenged.¹⁸

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 offensive 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,^{27,28} 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 an antipersonnel weapon were tested on Gruinard Island near the coast of Scotland in 1942. These experiments successfully produced anthrax among targeted sheep.²⁹ The island was quarantined because of focal soil contamination by *B anthracis* spores. The antipersonnel weapons were not mass produced, and neither the cattle cakes nor the explosive munitions were used.¹⁶ Great Britain continued research and development after the war in conjunction with the United States and Canada and performed secret open-air tests using pathogens in open ocean near the Bahamas and Scotland in 1948, 1952, 1953, 1954, and 1955. Simulant studies were performed off the coast of the United Kingdom in

1957, 1958, 1964, and 1965.¹⁶ Great Britain's offensive program was ultimately terminated between 1955 and 1956³⁰ because of budgetary constraints and reli-

ance on nuclear deterrence.^{27,28} Gruinard Island was decontaminated in 1986 using 2,000 tons of seawater and 280 tons of formaldehyde.³¹

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 unfeasible and ineffective. In the fall of 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 that the United States should reduce its vulnerability.

President Roosevelt established the War Reserve Service (with George W Merck as director) to develop defensive measures against a biological attack. 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. Cattle cakes using *B anthracis* spores were produced at Camp Detrick and shipped to Great Britain but were never used. No agents were produced at the Terre Haute plant because of safety concerns; simulant tests had disclosed contamination after trial runs. The War Reserve Service was disbanded after the war, and the Terre Haute plant was leased for commercial pharmaceutical production.²⁶ In January 1946 Merck reported to the secretary of war that although the focus of the program was to

defend against a biological threat, the United States clearly 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. At the end of the war, in a move that has now become controversial, Ishii, then a lieutenant general, and his fellow scientists were given amnesty for providing information derived from years of biological warfare research.¹⁹

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 the Soviets might resort to biological warfare resulted in expansion of the US program. A large-scale production facility in Pine Bluff, Arkansas, was established. The new 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 (see Table 1-1) in 26 years.³² However, the US military has never used biological weapons. The Central Intelligence Agency developed weapons using toxins including cobra venom and saxitoxin for covert operations; all records regarding their development and deployment were destroyed in 1972.³³

Field tests were done 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

TABLE 1-1
BIOLOGICAL AGENTS PRODUCED BY THE US MILITARY (DESTROYED 1971-1973) *

Lethal Agents	Incapacitating Agents	Anticrop Agents
<i>Bacillus anthracis</i>	<i>Brucella suis</i>	Rice blast
<i>Francisella tularensis</i>	<i>Coxiella burnetii</i>	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.

Proving Ground, Utah; remote Pacific Ocean sites; and populated areas including Minneapolis, Minnesota; St. Louis, Missouri; Eglin Air Force Base, Florida; New York, New York; and San Francisco, California. These studies tainted the history of the offensive biological warfare program. The Special Operations Division at Camp Detrick conducted most of the field tests as studies on possible methods of covert attack 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.^{32,34} In conjunction with the US Department of Agriculture, several open-air tests were conducted using anticrop agents at sites selected for safety.^{34,16} Open-air releases of human pathogens (*Coxiella burnetii*, *Francisella [Pasteurella] 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.^{35,34,16} 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.³⁶ Similar tests were conducted using the ventilation system of the New York City subway 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.³² 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.³⁷ 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.³² Public disclosure of the testing program in the *Washington Post* on December 22, 1976, and in US Senate hearings in 1977³⁸ resulted in harsh criticism of the continued use of *S marcescens* as a simulant after the Stanford epidemic.³⁹ 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. 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 nosocomial outbreaks resulting from opportunistic pathogens of low virulence complicating the use of medical devices and surgical procedures in the setting of antibiotic selection pressure.³⁹

The US program developed and incorporated modern biosafety technology and procedures such as 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). 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 below those reported by other laboratories. There were 48 infections and no fatalities at the production and testing sites.³²

After 1954, the newly formed Medical Research Unit at Fort Detrick conducted studies independent of those done by the Chemical Corps to develop vaccines and therapy to protect against biological agents. Researchers began using human volunteers in 1956 as part of a congressionally approved program referred to as “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 volunteers, and participation was voluntary with the informed consent of the volunteer. The program concluded with the end of conscription in 1973.

KOREAN WAR AND COLD WAR ALLEGATIONS

During the Korean War (1950–1953), North Korean, Chinese, and Soviet officials made numerous allegations of US biowarfare use. The descriptions of

biowarfare in many of the allegations appear to be based on Chinese experiences during World War II with “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 World Health Organization 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.⁴⁰ Reviews of documents from former Soviet archives published by a Japanese newspaper in 1998 provide evidence that the allegations were deliberate and fictitious propaganda.^{41,42}

Numerous unsubstantiated allegations were made during the Cold War era. The Soviet Union accused the United States of testing biological weapons on Canadian Eskimos, resulting in a plague epidemic,⁴³ and of collaborating with Colombia in a biological attack on Colombian and Bolivian peasants.⁴⁴ The United States was also accused of planning to initiate an epidemic

of cholera in southeastern China⁴⁵ and of the covert release of dengue in Cuba.⁴⁶

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 location of the alleged attacks made intelligence investigations extremely difficult. Attacks were never witnessed by Western intelligence operatives, 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.⁴⁷

DISARMAMENT

In July 1969 Great Britain issued a statement to the UN Conference of the Committee on Disarmament calling for the prohibition of development, production, and stockpiling of bacteriological and toxin weapons. That September the Soviet Union unexpectedly recommended a disarmament convention to the UN General Assembly. In November 1969 the World Health Organization 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. 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. Research was strictly directed to the development of vaccines, drugs, and diagnostics

as defensive measures. The UN then developed the 1972 Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological and Toxin Weapons and on their Destruction (1972 Biological Weapons Convention [BWC]), which prohibited any malicious research, production, or use 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.” The United States ratified both the 1925 Geneva Convention and the 1972 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 lacking⁴⁸; numerous attempts to formulate such measures have been unsuccessful because of numerous political, security, and proprietary issues.^{49,16} Only one allegation has been formally registered under the BWC: in July 1997 Cuba accused the United States of a biological attack with a crop pest insect, *Trips palmi*. The allegations were unsubstantiated in a BWC consultation that was concluded in December 1997.¹⁶ 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.

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 agent on targeted populations were still conjectural and for ethical and public health

reasons could not be empirically validated. Biological weapons were considered 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.^{50,16}

The US Army, in response to the 1969 presidential directive, did not await the BWC or its ratification. By May 1972 all personnel-targeted 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. Biological weapons have never been used by the US military. The Central Intelligence Agency destroyed its toxin samples per presidential orders after a US Senate investigation.³³ Fort Detrick and other installations involved in the offensive weapons program were redirected. In 1969 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. USAMRIID replaced the US Army Medical Unit.

THE SOVIET PROGRAM

Although a signatory to the 1925 Geneva Convention, the Soviet Union began its weapons development program at the Leningrad Military Academy in Moscow 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 during the pre-World War II era at Slovetky Island in the Baltic Sea and nearby concentration camps. The program subsequently expanded to include work with Q fever, glanders, and melioidosis, and possibly tularemia and plague. Outbreaks of Q fever among German troops in the Crimea and tularemia among the German siege forces of Stalingrad are two suspected, but unconfirmed, Soviet uses of biological warfare during World War II.⁵¹

During World War II 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 with Japanese plans. When Stalin died in 1953, a struggle ensued for control of the Soviet Union. Beria was executed during the power struggle, and Khrushchev, the new Kremlin leader, transferred the biological warfare program to the Fifteenth Directorate of the Red Army. Colonel General Yefim Smirnov, who had been the chief of army medical services during the war, became the director.⁵¹

Smirnov, who had also been Stalin's minister of health, was a strong advocate of biological weapons. In 1956 Defense Minister Marshall Georgi Zhukov announced that Moscow 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, the Soviets appeared to have subsequently in-

creased their biowarfare efforts.⁵² The Soviets doubted US compliance with the convention, which further motivated their program.⁵¹ The Soviet biological weapons effort became an extensive program, comprising various institutions under different ministries and the commercial facilities collectively known as Biopreparat. The Soviet Politburo had formed and funded Biopreparat to carry out offensive research, development, and production under the label of legitimate civil biotechnology research. Biopreparat conducted its clandestine activities at 52 sites and employed over 50,000 people. Annualized production capacity for weaponized smallpox was 90 to 100 tons.⁵⁰

The former Soviet Union was an active participant in the World Health Organization'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 a biological weapon. The World Health Organization announced the eradication of smallpox in 1980, and the world rejoiced at the elimination of a disease that had caused more human deaths than any other infection. The bioweapon developers in the former Soviet Union had a more cynical reason to celebrate. Smallpox eradication would result in the termination of vaccination programs; 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 delivery using intercontinental missiles.⁵¹

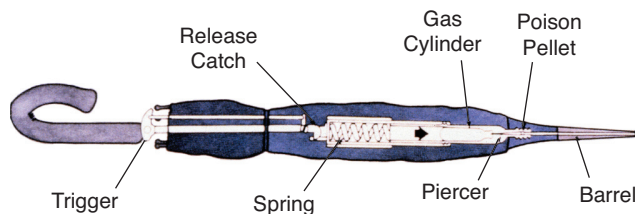


Fig. 1-1. An umbrella gun of this type was the clandestine weapon used to assassinate Bulgarian exile Georgi Markov in London in 1978. The weapon consisted of a spring-loaded piston, which would drive a carbon dioxide cartridge forward into a firing pin. The gas would then propel a poison projectile out of the hollow tip of the umbrella gun, through the clothing, and into the flesh of the intended victim. Reproduced from van Keuren RT. *Chemical and Biological Warfare, An Investigative Guide*. Washington, DC: Office of Enforcement, Strategic Investigations Division, US Customs Service; October 1990: 89.

In addition to military biological weapons programs, the Soviets developed toxin weapons for use by Warsaw Pact intelligence services. Perhaps the most dramatic example of assassination using a biological weapon occurred in September 1978 when Georgi Markov, a Bulgarian exile living in London, was attacked by a member of the Bulgarian secret service. A device concealed in the mechanism of an umbrella (Figure 1-1) surreptitiously discharged a tiny pellet into the subcutaneous tissue of his leg. He died mysteriously several days later with fever, hypotension, and clinical sepsis. The pellet (Figure 1-2), 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 after such a small dose.⁵³ That August in Paris, Vladimir Kostov, a Bulgarian defector living in Paris, had been attacked in a similar manner. He experienced pain and bleeding at the wound site and a fever, yet had no further complications. After hearing of Markov's death in September, he sought medical evaluation; X-ray radiographs disclosed a small metallic pellet in the skin. The pellet was surgically recovered from subcutaneous fat. Kostov then

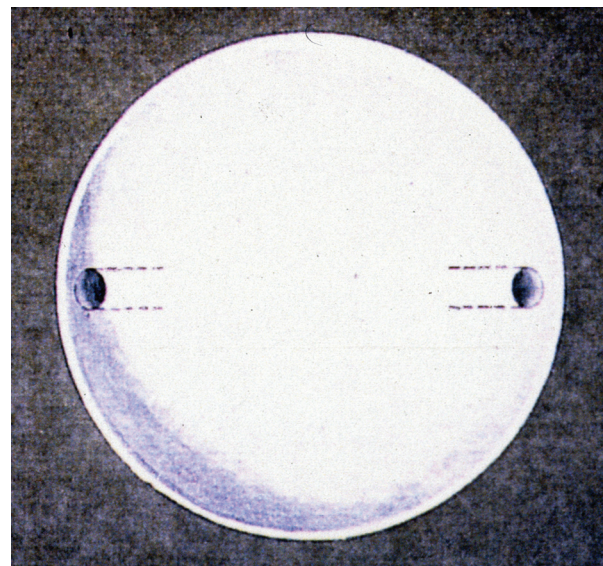


Fig. 1-2. A pellet of this type, designed to contain ricin toxin, was used to assassinate Georgi Markov in London and in the attempt on the life of Vladimir Kostov in Paris. The tiny, platinum-iridium pellet—the size of the head of a pin (0.068 in. diameter)—was cross-drilled with 0.016-in. holes in which ricin (or another toxin) could be placed. Reproduced from van Keuren RT. *Chemical and Biological Warfare, An Investigative Guide*. Washington, DC: Office of Enforcement, Strategic Investigations Division, US Customs Service; October 1990: 90.

tested positive for antiricin antibodies, supporting the probable use of ricin in these attacks.¹⁸

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 moved into the hospitals in Sverdlovsk and took control of the care of thousands of patients with a highly fatal form of anthrax. Suspicions emerged about an accidental release of anthrax agent into the urban area in the vicinity of a Soviet military installation, Compound 17.⁵⁴ The Central Intelligence Agency sought the opinion of Harvard biologist Matthew Meselson on the situation. Meselson had been a strong proponent of the Nixon ban on the US biological warfare program, and he attempted to refute 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 event generated enough concern within the Reagan administration and the Department of Defense to increase military biopreparedness.⁵⁴

Debate about the incident raged for the next 12 years. Meselson testified before the US Senate that the burden of evidence was that the anthrax outbreak resulted from the Soviets' failure to keep anthrax-infected animals out of the civilian meat supply, and not the consequence of an accident at a military weapons facility, as maintained by many US officials. Meselson asserted his opinion that the 1972 BWC had been a total success and no nation possessed a stockpile of biological weapons. In June 1992, during a brief but open period of detente, Meselson was allowed to take a team of scientists to review autopsy material and other evidence from the Sverdlovsk incident. After examining autopsy specimens of mediastinal tissue, team pathologist David Walker determined the disease had been contracted from inhalation of

anthrax spores, not from ingestion of tainted meat as the Soviets continued to allege.⁵⁴ The team's attempts to review hospital records of cases from the outbreak were unsuccessful because the records had been confiscated by the KGB. However, the team 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 a Soviet military microbiology facility during the first week of April 1979. A review of 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.⁵⁵

Russian leader Boris Yeltsin admitted in private conversations with President George H. Bush early in 1992 that the KGB and military had misrepresented the anthrax deaths. Subsequently, in a press release, Yeltsin admitted to the offensive program and the true nature of the Sverdlovsk biological weapons accident.⁵⁴ 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 Sverdlovsk epidemic.⁵⁶ Soviet defectors, including Ken Alibek, first deputy chief of Biopreparat from 1988 to 1992, confirmed that not only was the Sverdlovsk anthrax 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. In September 1992 Russia signed an agreement with the United States and Great Britain promising to end its weapons program and to convert its facilities for benevolent scientific and medical purposes.^{16,51,57}

SOUTH AFRICA

The South African Defense Force is alleged to have begun a small-scale biological weapons program in the early 1980s, primarily investigating *B anthracis* and *V cholerae*. The agents allegedly were used, but details

are not available. The program was closed in 1993 after diplomatic interventions by the United States and the United Kingdom, coincident with the demise of the apartheid regime.¹⁶

THE SPECIAL CASE OF IRAQ

The most ominous threat of biological warfare that US military forces have faced came during Operations Desert Shield and Desert Storm in 1990 and 1991. Intelligence reports suggested that Iraq had operated a

biological weapons program. Coalition troops trained in protective gear and stockpiled ciprofloxacin for use as postexposure prophylaxis against anthrax. Approximately 150,000 US troops received the Food and Drug

TABLE 1-2
BIOLOGICAL AGENTS PRODUCED BY IRAQ*

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

*Disclosed by the Iraq government after 1995
L=Liter

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 UN Special Commission on Iraq (UNSCOM) were confounded by misinformation and obfuscation. After General Hussein Kammal defected in 1995, the Iraqi government disclosed that it had operated a robust biological weapons program at six major sites since the 1980s. 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). These weapons were deployed but not used.^{58,59} The reasons behind Saddam Hussein's decision not to use these weapons are unclear; perhaps he was concerned about provoking massive retaliation. Alternately, 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.^{59,60}

The Iraqis claimed to have destroyed their biological arsenal immediately after the war but were unable

TABLE 1-3
DELIVERY SYSTEMS FOR BIOLOGICAL AGENTS DEVELOPED BY IRAQ*

Aerial Bombs		Missile Warheads	
Botulinum	100	Botulinum	13
<i>Bacillus anthracis</i>	50	<i>Bacillus anthracis</i>	10
Aflatoxin	16	Aflatoxin	2

*Disclosed by the Iraq government in 1995

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. The UNSCOM inspectors never received full cooperation from the Saddam Hussein regime, and they were ejected from Iraq in 1998. International concern led to renewed inspections in 2002 under UN Security Council Resolution 1441. The Iraqi government failed to cooperate fully with the inspections, and coalition forces invaded Iraq in 2003. In 2005, the Iraq Survey Group (an international group composed of civilian and military persons) 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, Hussein had perpetuated ambiguity regarding a possible program as a strategic deterrent against Iran.⁵⁷ 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 2003.⁶¹

BIOLOGICAL TERRORISM

Bioterrorism refers to use of biological agents by a political or religious group or cult (a group not otherwise recognized as an extension of the government of a state) to achieve a political or ideological objective. Bioterrorist incidents have increased markedly since 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.^{62,63}

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.¹⁸

The first documented incident of Rajneeshee use of a biological agent occurred on August 29, 1984. Two Wasco County commissioners visiting Rajneeshpuram 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. As a consequence of this attack, 751 cases of enteritis resulted in at least 45 hospitalizations.^{18,64}

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 \$300,000,000 in financial assets. Aum Shinrikyo mimicked the organization of the Japanese government with "ministries and departments." "Health and welfare" was headed by Seiichi Endo, who had worked in genetic engineering at Kyoto University's viral research center. "Science and technology" was headed by Hideo Murai, who 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.¹⁸

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 to disseminate the anthrax. 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 Ebola virus, which the cult claimed to have cultivated. 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 Yokohama, Yokusuka 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 same month, the cult spread anthrax 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 anthrax, 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. Ultimately Aum Shinrikyo gave up on its biological weapons and released sarin in the Tokyo subway on March 20, 1995.¹⁸

Reasons given for the cult's failure include its use of a nontoxin-producing (or low yield) strain of *C botulinum*, use of a low-virulence 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.¹⁸

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,⁶⁵ and he threatened to attack Las Vegas with the *B anthracis*. His case led to the development of stringent regulations for the procurement and shipping of select microbes.

During the late 1990s the US government launched an ambitious program to enhance biological preparedness at local, state, and federal levels,⁶⁶ 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. The Department of Health and Human Services (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. A national stockpile of key pharmaceutical agents and vaccines, now called the Strategic National Stockpile, was prepared. The Laboratory Response Network for Bioterrorism, also managed by CDC, provided coordination of testing, sample shipment, and communication between designated local, regional, and reference laboratories. Department of Defense 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.

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.⁶⁷

Stevens was admitted to a Palm Beach, Florida, hospital with high fever and disorientation on October 2, 2001. By October 5, 2001, Stevens was dead from inhalational anthrax, the first such case in the United States in over 20 years. An autopsy revealed hemorrhagic pleural effusions and mediastinal necrosis. 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. US postal facilities in the national capital area and in Trenton, New Jersey, were also contaminated.

At least five letters (four recovered) and, possibly, as many as seven letters containing anthrax spores had been mailed, possibly in two mailings, on September 18, 2001, and October 9, 2001. Twenty-two people contracted anthrax, with 11 inhalational cases resulting in five deaths. Screening and postexposure prophylaxis resulted in significant disruption of operations at the Hart US Senate Office Building and in US postal facilities. Millions of dollars were spent on environmental decontamination. Public alarm was compounded by numerous “white powder” hoaxes.

A significant lesson learned from this incident was the importance of effective and accurate communication regarding the nature of the threat and response efforts. Farsighted emergency planning and training, in addition to the integration of federal and local medical, public health, and law enforcement agencies, were essential in the response to the 2001 anthrax mailings. These preparations in New York City and other cities enabled an unprecedented public health response. The Laboratory Response Network and military laboratories such as USAMRIID processed over 125,000 clinical specimens and 1 million environmental samples. USAMRIID ran over 260,000 assays on over 30,000 samples in 9 months. Prophylaxis supplied from the national stockpile was offered to nearly 10,000 individuals at risk. There were no cases among prophylaxis recipients.^{68,69} Anthrax treatment guidelines advocating multidrug antibiotic combinations and aggressive intensive care were disseminated,⁷⁰ and the case fatality rate for inhalational anthrax, historically exceeding 90%, was reduced to 45%.^{71,72} Bioterrorism response has since been strengthened with additional infrastructure and linkages among the emergency response, public health, clinical, and laboratory sectors.^{68,69}

Since the fall of 2001, much has been accomplished to better prepare the nation for the threat of bioterrorism. 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 the Department of Defense. 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.

The Office of Public Health Emergency Preparedness at DHHS, formed after the 2001 anthrax attacks, began to coordinate civilian medical countermeasure development by the National Institute of Allergy and Infectious Diseases, CDC, and the Department of Defense, under the leadership of eminent scientists and physicians such as DA Henderson and Philip K Russell. On July 21, 2004, President Bush signed legislation creating Project Bioshield, 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. In the past 4 years, new medical countermeasures added to the Strategic National Stockpile include a new cell culture-derived smallpox vaccine; vaccinia immune globulin to counteract smallpox vaccine side effects; significantly increased doses of botulinum antitoxins to treat casualties of botulinum poisoning; antibiotic stocks for anthrax, tularemia, and plague treatment; and ventilators for respiratory support. Furthermore, DHHS has planned for the stockpiling of the licensed anthrax vaccine, a new recombinant anthrax vaccine, more doses of botulinum antitoxins, a safer smallpox vaccine that can be given to immunocompromised individuals, and anthrax adjunctive therapies.

CDC launched a comprehensive smallpox preparedness program in 2002 as a result of concern about the potential use of *Variola* as a biological agent. The program integrated community, regional, state, and federal healthcare and public healthcare organizations and featured logistical preparation; training and education; risk communication; surveillance; and local preparations for mass vaccination, isolation, quarantine, and humane treatment of patients in designated facilities.⁷³ A strategy was adopted based on preexposure vaccination of carefully screened and trained members of first-response teams, epidemiological response teams, and clinical teams at designated facilities. Over 400,000

selected military personnel and 38,000 civilian emergency responders and healthcare workers in designated smallpox response teams were vaccinated. The program calls for a "ring vaccination" strategy: identifying and isolating cases, with postexposure vaccination and active surveillance of those potentially exposed by the initial release. Vaccinated individuals are to be monitored under active surveillance. Patients with suspected or confirmed smallpox are to be grouped together and quarantined in designated buildings (Category X for suspected cases, Category C for confirmed cases) with independent ventilation systems.⁷³ Researchers studied the immunogenicity of diluted vaccine because of shortages of vaccine supplies (approximately 15 million doses). A 10-fold dilution was found to be immunogenic; diluting the existing vaccine by 5-fold to 10-fold was considered an emergency measure.⁷⁴ Contracts for the production of a new cell culture-derived vaccine were awarded in 2000; CDC now holds sufficient cell culture-derived vaccine for the entire US population.⁷³ Severe adverse reactions have been rare during the smallpox preparedness program. However, cases of myocarditis and sporadic cardiovascular events among patients with vascular risk factors led to additional exclusion criteria.⁷⁵ The search for a less reactogenic vaccine has rekindled interest in a highly attenuated vaccinia strain (Modified Vaccinia Ankara)⁷⁶ and has led to the development of a DNA subunit vaccine candidate.⁷⁷

The threat of bioterrorism continues. 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.⁷⁸

US forces operating in northern Iraq in 2003 seized a camp linked to Al Qaeda reportedly containing instructions and equipment for ricin extraction.^{79,80} Meanwhile, a raid on a London apartment yielded a written formula for ricin production, its natural source (castor beans), and a suitable solvent (acetone) for its extraction. Although tests for ricin were negative,⁸¹ one of the tenants, an Al Qaeda-trained operative, was convicted of plotting a ricin attack. He planned to contaminate hand rails in the railway system connecting London and Heathrow Airport.⁸² In March 2003 two flasks containing ricin were discovered in a railway station in Paris.⁸³ Ricin-containing packages mailed to officials in South Carolina, the White House, and the US Senate were intercepted during 2003 and 2004.^{84,85} No casualties or significant environmental contamination were related to these incidents.

BIOCRIMES

Biocrime refers to 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,⁸⁶ 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 Tucker⁶⁵ produced 29 deaths and 31 injuries.

Biocrimes are typically attempted by perpetrators with scientific or medical expertise or who have recruited suitably trained accomplices. 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,⁸⁶ the biological agents were usually obtained from a legitimate source or stolen; the perpetrators produced agent in only two cases. 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.

Numerous and highly varied biocrimes have been reported; only several representative examples can be included in this chapter. The works of Tucker,⁶⁵ Carus,^{18,86} and Leitenberg¹⁶ provide comprehensive descriptions and analysis.

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; these crimes resulted in over 120 cases of infection and four deaths. Dr Suzuki was reportedly motivated by his dissatisfaction with the medical training system and a desire to further his research on typhoid fever.¹⁸

In 1995 Dr Debra Green pleaded no contest to charges of murder and attempted murder. The murder charges stemmed from the deaths of two of her children in a fire thought to have been caused by arson. The attempted murder charges stemmed from the poisoning of her estranged husband with ricin. Green was sentenced to life imprisonment.¹⁸

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.⁸⁷ 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.¹⁸

Murders by direct injection included the use of diphtheria toxin in Russia in 1910 and *Y pestis* in India in 1933. The director of a Norwegian nursing home was convicted in 1983 of murdering 22 patients by injecting them with a curare derivative. Biocriminals have also harnessed the most lethal emerging pathogen of the 20th century; there have been at least four murder attempts by injecting victims with human immunodeficiency virus-infected blood.¹⁸

SUMMARY

The history of state-sponsored biological weapons programs is obscured by secrecy, propaganda, and a lack of rigorous microbiologic or epidemiological data to confirm allegations of use. With the exceptions of German sabotage during World War I, the Japanese field trials during World War II, and state-sponsored assassination by espionage agents, there are no well-documented or confirmed biological attacks by nation-states. In retrospect, the public health disaster at Sverdlovsk and political consequences after disclosures suggest that the liabilities resulting from state-sponsored biological weapons programs have outweighed potential strategic advantages. Biological weapons programs have been renounced by over 140 signatory states to the 1972 BWC for numerous political and

strategic considerations. However, recent disclosures regarding the former Soviet program and findings by UNSCOM and the Iraq Survey Group underscore the ambitious intent and potential realization of covert state-sponsored programs. Furthermore, the Sverdlovsk accident provided a lethal "proof of concept" of what follows an airborne release of highly refined agent. According to an unclassified US Department of State report in 2005, nations suspected of continued offensive biological warfare programs in violation of the BWC include China, Iran, North Korea, Russia, Syria, and possibly Cuba. Counter-proliferation efforts, including verification of compliance of signatory states to the convention, remain an ongoing challenge.

The threat of bioterrorism reached paramount

importance in October 2001 and continues to present a formidable challenge. Increasingly, these terrorist organizations have taken an interest in biological agents.⁸⁸ One of the more alarming recent trends has been the increased motivation of terrorist groups to inflict mass casualties. Most biological incidents have been hoaxes, which have nonetheless resulted in considerable mayhem. Attacks with agent have usually been unsuccessful. Even the technically advanced program of the Aum Shinrikyo was a failure, most likely because of technical challenges posed by constructing an effective aerosol generator or other delivery devices. The likelihood of amateurs using homemade equipment to successfully launch a biological weapon of mass destruction is remote. Terrorists still rely on simple yet effective explosives as their weapon of choice. However, events in Iraq, the United Kingdom, and the United States reveal continued intent. The discovery of Al Qaeda laboratories in Afghanistan demonstrates a concerted effort to harness

modern technology for malicious purposes. The possibility of a major bioterrorist attack resulting in massive casualties cannot be ignored. Medical personnel, public health officials, and government agencies that deal with emergency response must be prepared.

A coordinated response integrating local and federal intelligence, law enforcement, public health, and medical assets affords a measured response based on risk analysis (credibility of the attack, results of rapid identification tests); postexposure surveillance; prophylaxis; treatment of casualties; and risk communication. This coordinated response confers a capability to mitigate the clinical public health consequences of attacks and rapidly defuse hoaxes and obviate social mayhem. The response to the anthrax mailings of 2001 demonstrated that although it is impossible to prevent all biological casualties, much can be done to minimize the morbidity, mortality, and social disruption of an intentional epidemic.

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Chapter 2

FOOD, WATERBORNE, AND AGRICULTURAL DISEASES

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INTRODUCTION

FOODBORNE AND WATERBORNE PATHOGENS AND DISEASES

PATHOGEN SUMMARY

WATER SUPPLY CONCERNS

AGRICULTURAL TERRORISM

FOOD AND WATER SECURITY

SUMMARY

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INTRODUCTION

Food and waterborne pathogens cause a considerable amount of disease in the United States. A decade ago, the US Department of Agriculture (USDA) estimated that medical costs and productivity losses for diseases caused by the five leading foodborne pathogens are as much as \$6.7 billion per year.¹ 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 are stable in food products or potable water. These agents include (a) *Clostridium botulinum* toxin, (b) the hepatitis A virus, (c) *Salmonella*, (d) *Shigella*, (e) enterohemorrhagic *Escherichia coli* species, (f) *Cryptosporidium parvum*, (g) *Campylobacter jejuni*, (h)

Listeria monocytogenes, and (i) *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. The potential for nonlisted biological agents such as mycotoxins and parasites (eg, *Taenia* sp) to be used in a bioterrorist event also should 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 the nation's food-to-farm continuum (agricultural terrorism). For a more extensive review of these topics, readers may consult more specialized texts on food² and waterborne³ diseases and agricultural terrorism.^{4,5}

FOODBORNE AND WATERBORNE PATHOGENS AND DISEASES

B anthracis is the causative agent of two forms of foodborne anthrax: (1) oropharyngeal and (2) gastrointestinal. Although *B anthracis* would cause the most potential harm via an aerosol release, anthrax 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.⁶ However, given that the early diagnosis of gastrointestinal anthrax is difficult and problematic 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.⁷

C botulinum is the causative agent of botulism intoxication, of which there are three natural manifestations: (1) classic, (2) wound, and (3) infant botulism. A bioterrorism use of botulinum toxin would possibly occur through inhalational intoxication, as was considered by the Aum Shinrikyo cult in Japan.⁸ *C botulinum* produces the most potent natural toxin known; the human lethal dose of type A toxin is approximately 1.0 $\mu\text{g}/\text{kg}$.⁹ 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 preparation¹⁰ and home canning¹¹ and pickling.¹² 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.¹³

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%.¹⁴ 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.¹⁵

A recent controversial paper¹⁶ explored the potential for botulinum toxin contamination of the milk supply. A 9-stage cows-to-consumer supply chain was examined, which accurately reflected 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 >1 gram of toxin, and 10 grams would affect about 568,000 milk consumers.¹⁶ The National Academy of Sciences published this information to foster further discussion and alert authorities to the dangers to the milk supply from purposeful contamination.¹⁷ The paper describes interventions that the government and the dairy in-

dustry could take to prevent this scenario. Officials at the US Department of Health and Human Services requested that this paper not be published. Regardless, publication ensued because the The National Academy of Sciences was convinced that this information would not enable bioterrorists to conduct an attack, and that the paper itself would stimulate biodefense efforts. However, whether this information presents a "roadmap for terrorists" by exposing vulnerabilities in food processing remains to be determined.¹⁸

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 restricted. *C jejuni* is the most commonly reported bacterial cause of foodborne infection in the United States. Chronic sequelae associated with *C jejuni* infections include Guillain-Barre syndrome¹⁹ and arthritis.²⁰ 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.²¹ Reservoirs for *C jejuni* include wild fowl and rodents.²² The intestines of poultry are easily colonized with *C jejuni*,²³ and it is a commensal inhabitant of the intestinal tract of cattle.²⁴ Antibiotic resistance of *Campylobacter* is a growing concern for poultry.²⁵ *Campylobacter* has a 100 to 1,000 cell infective dose, with poultry being the primary source of infection in the United States.²⁶ Insect transmission by several fly species has also been documented.²⁷ 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.²⁸

Salmonellosis is the second most common foodborne illness,²⁹ and contaminated food is the principal route of disease transmission.³⁰ There are over 2,400 *Salmonella* serotypes, many of which can cause gastroenteritis, manifested as diarrhea, abdominal pain, vomiting, fever, chills, headache, and dehydration. Other diseases from *Salmonella* infections include enteric fever, septicemia, and localized infections. Poultry is a principal reservoir of the salmonellae. Water, shellfish, raw salads, and milk also are commonly implicated as vehicles for this pathogen. In humans, the most highly pathogenic *Salmonella* species is *S typhi*. This bacterium is the causative agent of typhoid fever, which comprises about 2.5% of salmonellosis in the United States. The symptoms of typhoid include septicemia, high fever, headache, and gastrointestinal illness.

An immense outbreak of milk-borne salmonellosis from *Salmonella enteritica* serovar *typhimurium* occurred in northern Illinois in 1985, with more than 14,000 peo-

ple reported ill and five deaths.^{31,32} A nonpurposeful outbreak of this magnitude demonstrates what could be initiated by bioterrorism. Cases also were reported in the neighboring states of Indiana, Iowa, and Michigan because the contaminated milk was distributed via supermarket distribution systems.³³ Medical treatment was complicated because the strain of *S typhimurium* was found to be resistant to antibiotics. The cause of the outbreak was the accidental comingling of raw milk into the pasteurized product in the milk plant.³⁴

The earliest use of biological weapons by the Japanese during World War II was the intentional poisoning of wells with *S typhimurium* along the Russian border of Mongolia in 1942.³⁵ 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.³⁶ The size and nature of this outbreak helped to initiate a criminal investigation, which previously was almost never 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.³⁷ In October 1985 authorities found an opened vial holding the original culture type of *S typhimurium* in the Rajneeshee clinic laboratory.

Listeria monocytogenes is often found in silage, water, and the environs of animal fodder.³⁸ Soft cheeses,³⁹ raw or contaminated milk,⁴⁰ and contaminated refrigerated foods⁴¹ 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.⁴² Fetuses, the newborn,⁴³ the elderly,⁴⁴ and those immunocompromised⁴⁵ 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.⁴⁶ The milk originated from a group of farms at which listeriosis occurred in dairy cows. Of the 49 infections associated with this outbreak, 14 patients died.

E coli O157:H7 produces two verotoxins and has emerged as a major cause of serious pediatric illness. 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.⁴⁷ Children younger than 5 years of age are at greatest risk for hemolytic uremic syndrome when

infected with *E coli* O157:H7 or other enterohemorrhagic *E coli* species, and deaths from these infections occur most often in the age ranges of 1 to 4 years and 61 to 91 years.⁴⁸

A major source of EHEC exposure is from consumption of and contact with beef cattle.⁴⁹ About 20% of the ground beef consumed in the United States is derived from cull dairy cattle, which may be an important contributor to this bacterial contamination of the food supply.⁵⁰ For example, during July 2002, the Colorado Department of Public Health and Environment identified an outbreak of *E coli* O157:H7 infections, which linked 28 illnesses in Colorado and six other states to the consumption of contaminated ground beef products. Seven patients were hospitalized; five developed hemolytic uremic syndrome.⁵¹ *E coli* contaminated food items commonly result from use of cattle waste for fertilizer, or coming into contact with cattle products. Outbreaks have occurred from exposure to various *E coli*-tainted food items, including alfalfa⁵² and radish⁵³ sprouts, parsley,⁵⁴ lettuce,⁵⁵ apple cider,⁵⁶ unpasteurized gouda cheese,⁵⁷ raw milk,⁵⁸ recontaminated pasteurized milk,⁵⁹ and salami,⁶⁰ as well as through petting zoos⁶¹ and environmental transmission.^{62,63} Waterborne outbreaks with *E coli* O157:H7 also occur, thereby demonstrating the potential for such contamination from a purposeful effort. 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.⁶⁴ Swimming water-associated outbreaks of *E coli* O157:H7 also have occurred.^{65,66}

Humans are the major reservoir for *Shigella* and the primary source of subsequent infections. It is thought that worldwide *Shigella*-associated illness causes about 165 million cases per year, of which fewer than 1% occur in industrialized nations.⁶⁷ *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. Four serogroups (A through D) cause approximately 80% of shigellosis cases in the United States. Immunity is serotype-specific,⁶⁸ vaccine development has been problematic,⁶⁹ and the species can easily become resistant to antibiotics.⁷⁰ Infants and young children are most susceptible to shigellosis, attributable in part to toiletry behaviors and child care practices. Although not an environmentally hardy organism, *Shigella* is highly infectious and can be very persistent in a close community environment.⁷¹ The infectious dose for *Shigella* is from 10 to 100 organisms, and *Shigella* contamination can cause outbreaks associated with food, water, and milk. Shigellosis also has been associated with recreational swimming.⁷² Shigellosis is readily

transferred from person-to-person contact and through fomites;⁷³ it can also be transmitted by insect vectors (primarily flies).⁷⁴ 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 in other enteric pathogens such as *Salmonella* and *V cholerae*.

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.⁷⁵⁻⁷⁹ Seroprevalence surveys indicate that about 20% of the US population have been infected with *Cryptosporidium* by adulthood.⁸⁰ 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, alternatively, 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.⁸¹ The precise infectious dose is unknown; research indicates that a range of 9 to 1,024 oocysts will initiate infection.⁸² The pathobiology is not completely known; 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.⁸³ A significant reservoir worldwide for *Cryptosporidium parvum* is domestic livestock, predominately cattle.⁸⁴ Drinking-water outbreaks have affected as many as 403,000 individuals in a 1993 outbreak in Milwaukee.⁸⁵ The water in the Milwaukee system was both filtered and chlorinated.⁸⁶ This organism's resistance to chlorine treatment ensures that it will remain a concern in treated potable water,⁸⁷ and therefore a risk to immunocompromised individuals for whom this organism causes severe and chronic life-threatening gastroenteritis.⁸⁸

Humans are the source of the *Hepatitis A virus*. Illness caused by hepatitis A is characterized by sudden onset of fever, malaise, nausea, anorexia, and abdominal discomfort, followed by jaundice. The infectious dose is not precisely known but is thought to be 10 to 100 virus particles. 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.⁸⁹ Permanent immunity to hepatitis A is assumed subsequent to infection⁹⁰ or immunization completion.⁹¹ The advent of nationwide hepatitis A vaccination programs is gradually causing a decrease in disease incidence and susceptible population.⁹² As a result of these successful immunization programs, hepatitis A may in time cease to be a public health concern.⁹³

The potential for hepatitis A virus transmission in drinking water was demonstrated in the hepatitis A outbreak among members of the varsity football team at the College of the Holy Cross in Worcester, Massachusetts, in 1969. 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).⁹⁴ Because of this discrepancy, the illness may have been caused by another pathogen present in the water. 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.^{95,96}

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.^{97,98} Although they are not on the CDC threat list, mycotoxins (including aflatoxin B1, ochratoxin, T-2 toxin, deoxynivalenol [DON], and nivalenol [NIV], and others), often have oncogenic properties from chronic exposure, and may also have potential for use as small-scale biological weapons. 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, indicates that a ready substrate for growth is available and purposeful contamination of these foodstuffs is possible. Mycotoxicoses are often undiagnosed and hence unrecognized by public health authorities, except when large numbers of people are affected.⁹⁹ 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.¹⁰⁰

Large naturally occurring outbreaks of trichothecene intoxications have occurred, including an outbreak affecting 130,000 people in the Anhui province in China in 1991 caused by moldy wheat and barley. *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.^{101,102} A large exposure of trichothecene mycotoxin from moldy grain and bread in Orenburg, Russia, in 1944 caused alimentary toxic aleukia and subsequent mortality in at least 10% of the population.¹⁰³ Although outbreaks of mycotoxicoses have decreased greatly as a result of increases in hygiene measures, they still occur in developing countries,¹⁰⁴ are considered a serious international health problem,¹⁰⁵ and are also a risk for domestic animals.¹⁰⁵⁻¹⁰⁷

The history of mycotoxin use as a biological weapon includes efforts by Iraq's biological weapon program to develop and use aflatoxins during the 1980s. Strains of *Aspergillus flavus* and *A. parasiticus* were cultured, and 2,300 liters of concentrated toxin were extracted. This aflatoxin was used mostly to fill missile warheads, and the remainder was kept stockpiled.^{108,109} 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 purposeful¹¹⁰ or natural¹¹¹ events has never been completely resolved. These events indicate the potential for mycotoxin use as a biological weapon or bioterrorism agent.

Parasites such as tapeworms (eg, *Taenia* sp) may have the potential for use as agents of bioterrorism. It is conceivable that, for example, a culture of *Taenia solium* eggs be poured onto a salad bar or into water, and be ingested and cause illness. Symptoms of *taeniasis* from ingestion of the eggs would include cysticercosis, which would not appear for weeks to years following infection. However, this infection timeline should not eliminate parasites from consideration as having the potential for bioterrorist use. In their novel *The Eleventh Plague*, Marr and Baldwin present just such a scenario, with devastating effects.¹¹² *T. solium* has the potential to be transmitted from person-to-person through food handlers with poor personal hygiene, adding to the spread of the outbreak.¹¹³ 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.¹¹⁴

TABLE 2-1
FOOD AND WATERBORNE DISEASE PATHOGENS

Pathogen	Incubation Period	Infective or Toxic Dose*	Mortality in United States	Bloody Diarrhea
Enterohemorrhagic <i>Escherichia coli</i>	3–4 d	10–10 ²	rare	yes
<i>Salmonella typhi</i>	8–14 d	10–10 ²	low	yes
<i>Salmonella sp</i>	6–72 h	10 ² –10 ³	low	yes
<i>Shigella dysenteriae</i>	1–7 d	10–10 ²	rare	yes
<i>Campylobacter jejuni</i>	2–5 d	≥ 5 × 10 ²	rare	yes
<i>Clostridium botulinum</i> toxin	12–72 h	70 μg [†]	5%–10%	no
<i>Vibrio cholera</i>	2–3 d	10 ⁶	rare	no
<i>Cryptosporidium sp</i>	7 d	9–1,024	rare	no
<i>Listeria monocytogenes</i>	3 - > 90 d	unknown	high	no
<i>Hepatitis virus hepatitis A</i>	30 d	10–10 ²	low	no
Norovirus	1–2 d	< 10 ²	rare	no
Mycotoxins	mins–mos [‡]	4 mg/kg [§]	rare	yes

*The number of organisms unless otherwise noted.

†Oral lactate dehydrogenase₅₀ for a 70 kilogram human

‡Dose-dependent

§Oral lactate dehydrogenase₅₀ for laboratory rat

PATHOGEN SUMMARY

Table 2-1 categorizes various pathogens according to their threat potential 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. A high dose of *Salmonella* is required to cause illness. If the infectious or toxic dose required for illness from an organism is 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 demonstrated a reality concerning 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.¹¹⁵ 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 public¹¹⁶ if exacerbated by media coverage of the epidemic.¹¹⁷

WATER SUPPLY CONCERNS

Poisoning water supplies is one of the oldest methods of warfare. The earliest documentation of 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 to their stomachs and all lay unable to move."¹¹⁸ 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. The modern water

facility contains various treatment processes, including aeration, coagulation and flocculation, clarification, filtration, and chlorination.¹¹⁹ 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...¹²⁰ A terrorist might bypass the purification process and introduce a pathogen later in the distribution system. A private well water supply system may be more vulnerable because it may have a smaller volume of water and a less extensive purification system. Another potential avenue for purposeful waterborne contamination is the addition of a pathogen to a building's water supply, which would present an enclosed system, with likely little or no subsequent water treatment processes and a precise target community.

Waterborne pathogens included on the CDC threat list are *Vibrio cholerae* and *C parvum*. The Milwaukee outbreak with *C parvum* previously mentioned demonstrates the potential to affect great numbers of people with public water supply contamination. 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 state county fair.¹²¹ According to a comprehensive review of potable water threats by Burrows and Renner, potential water threat agents may also include *B anthracis*, *Brucella*, *V cholera*, *Clostridium 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.¹²² The hepatitis A outbreak that occurred at the College of the Holy Cross in 1969

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. Nongastroenteritis recreational water outbreaks often include those caused by *Pseudomonas aeruginosa*, *Naegleria fowleri*, and *Legionella*.¹²³ 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.¹²⁴ 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 those ill persons. The median age of the ill persons was 8 years old. 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.¹²⁵

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.¹²⁶ An example of a new program to detect purposeful contamination of the water supply is the WaterSentinel program.¹²⁷ However, much work remains to attain full biosecurity of the US water supply.^{128,129}

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 2-1). 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.

From 1952 to 1960, a tribal insurgency in British-controlled Kenya was known as the Mau-Mau, which is a Swahili acronym for "Let the white man go back abroad so the African can get his independence."¹³⁰ In 1952 the Mau-Mau used the indigenous poisonous African milk bush (*Synadenium compactum*) to kill 33 cows at a mission station.¹³¹

Anticrop terrorism has been claimed 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 outbreaks of the Colorado potato beetle occurred in England and the United States,

and Germany was suspected of releasing the insects. Germany conducted large-scale breeding and field trial dispersals of the insects in Germany, which may have backfired by initiating local crop infestations.^{132,133} An offensive research program was conducted at the Kruft Potato Beetle Research Station near Koblenz by Dr Martin Schwartz.¹³⁴ In 1950 Soviet-occupied East Germany accused the United States of releasing the Colorado potato beetle.¹³⁵ Other insect pests can wreak economic havoc upon crops. 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.¹³⁶ 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.¹³⁷

In the United States, livestock may be more susceptible to agroterrorism than crops (Figure 2-2). Because US disease eradication efforts among livestock herds have been so successful, much of the nation's

livestock is either unvaccinated or unmonitored for disease by farmers and veterinarians. Upon infection, livestock may become a vector¹³⁸ or reservoir¹³⁹ for disease transmission. This potential was plainly demonstrated with the outbreak of foot and mouth disease (FMD) in the United Kingdom in 2001.¹⁴⁰ This outbreak was the single largest FMD epidemic experienced in the world.¹⁴¹ Agricultural and food losses to the United Kingdom exceeded \$4.6 billion,¹⁴² and psychological effects in residents of the worst-affected areas were extensive and long-lasting.¹⁴³ The United States has not had an outbreak of this disease since 1929.¹⁴⁴ The USDA has developed national protective measures to prevent a reintroduction.¹⁴⁵ The relevance of 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. Consideration was given to aerial dissemination and dispersal of the FMD virus through contaminated hay and grass.¹⁴⁶ FMD is thought to in-



Fig. 2-1. Some of the greatest vulnerabilities from agricultural terrorism may exist at the farm end of the farm-to-food continuum.
Photograph: Courtesy of US Department of Agriculture, Washington, DC.

herently spread through airborne virus transmission, a problematic issue for outbreak containment.¹⁴⁷

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 also incidental costs would result from lost production, the destruction of potentially diseased products, and containment (including quarantine, drugs, and diagnostic and veterinary services). Much of the costs of these programs would be borne by the federal and state governments.¹⁴⁸ Export markets would be rapidly lost. 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.¹⁴⁹ 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,¹⁵⁰ rinderpest,¹⁵¹ and African¹⁵² and classical swine fever.¹⁵³

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 States¹⁵⁴ (some of these zoonotic pathogens also endanger humans and appear on the CDC Category A list)¹⁵⁵; these pathogens are listed separately by the USDA as overlap agents and toxins. Another USDA list enumerates harmful plant pathogens.¹⁵⁶



Fig. 2-2. Livestock may be more susceptible to agroterrorism than crops.
Photograph: Courtesy of US Department of Agriculture, Washington, DC.

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."¹⁵⁷ In American society, the farm-to-fork continuum, which includes production, processing, distribution, and preparation, has myriad potential vulnerabilities for natural and intentional contamination.¹⁵⁸ Centralized food production and widened product distribution systems present increased opportunities for the intentional contamination of food.¹⁵⁹

There are many opportunities along the food and water production continuum to accidentally or intentionally introduce various pathogens, many of which are not categorized as threat agents.¹⁶⁰ 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.

Studying incidents of nonpurposeful foodborne pathogen contamination may reveal potential avenues for purposeful outbreak scenarios. A 1985 Minnesota outbreak affecting more than 16,000 persons with antimicrobial-resistant salmonellosis was eventually thought to have been caused by cross-contamination of raw milk into a pasteurized milk product sold to the public.¹⁶¹ The potential for bioterrorist contamination of the milk supply is obvious. 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.

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.¹⁶² The responsibility for food safety and security throughout this 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 similarly share these responsibilities, including the Food and Drug Administration, the USDA, the Department of Health and Human Services, the US Public Health Service, the 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 have been recent 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 wants to cause harm without consideration of whom or how many people are affected. The target population may then define the vulnerabilities.

Strategies also should 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.

Although many production processes can receive much attention, a targeted focus is often on the inputs to food, water, or agricultural production. When a product leaves the plant, that attention may be discontinued. The time and route of delivery, as well as the security of the transportation, may be the most important with reference to vulnerability and should not be overlooked when security planning.

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

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 ani-

mal 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 3

EPIDEMIOLOGY OF BIOWARFARE AND BIOTERRORISM

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INTRODUCTION

THE EPIDEMIOLOGY OF EPIDEMICS

Definition

Recognition

Potential Epidemiological Clues to an Unnatural Event

Outbreak Investigation

EPIDEMIOLOGICAL CASE STUDIES

Bioterrorism Events

Accidental Release of Biological Agents

Studies of Natural Outbreaks for Potential Bioweapon Use

EPIDEMIOLOGICAL ASSESSMENT TOOL

IMPROVING RECOGNITION AND SURVEILLANCE OF BIOTERRORISM

SUMMARY

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INTRODUCTION

Preparing for and responding to biowarfare (BW) or bioterrorism (BT) falls squarely in the realm of public health and in the purview of public health professionals. Basic epidemiology is needed for management 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 2. 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, baseline rates of disease are needed to determine whether an epidemic occurs. This information is obtained at the hospital or community level, or at the state, national, or global level. As an example, thousands of influenza cases in January in the United States may not be unusual; however, thousands of cases in mid-July may be cause for concern. Also, even a single case of a rare disease can be considered an epidemic. With the absence of 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, viral hemorrhagic fevers, and plague (especially pneumonic), 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 3-1).

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 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 the characteristics of the three to control an epidemic. In one scenario, if potential hosts are vaccinated, disease spread would be significantly limited because of

herd immunity. However, if the environment is modified, spread may be limited; for example, cleaning up garbage around a home limits rat food and harborage, and thus reduces the likelihood of bringing fleas closer to potential human hosts, limiting a potential bubonic plague outbreak.³

Recognition

Immediate effects are evident when an explosion occurs or a chemical weapon is released. However, casualties produced after a BW/BT release may be dispersed in time and space to primary care clinics and hospital emergency departments because of the inherent incubation periods of the pathogens. Therefore, the success in managing a biological event hinges directly on whether and when the event is recognized.

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

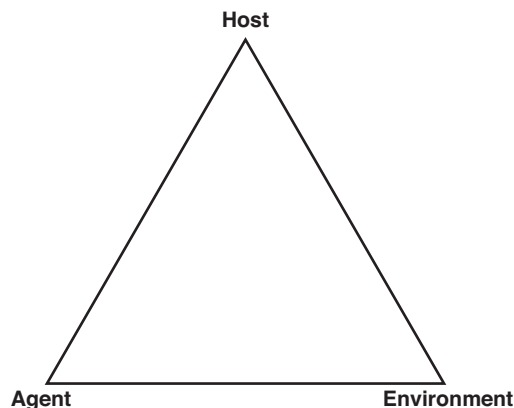


Fig. 3-1. The epidemiological triangle

35 deaths that led to a massive vaccination effort and border closure.⁴ Early disease recognition may have significantly modified the outcome. One modeling study of a BT-caused smallpox outbreak showed 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 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. Physicians are frequently taught to consider common illnesses first and might instead consider the source to be an endemic disease, a new or emerging disease, or a laboratory accident before considering BW/BT.⁶ Therefore, care providers should be familiar with the diseases of BW/BT and maintain a healthy “index of suspicion” to recognize an event early enough to significantly modify the outcome.⁷

Astute clinicians, hospital infection control personnel, school or healthcare facility nursing staff, laboratory personnel, and other public health workers 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 identification 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, doxycycline or ciprofloxacin) or orders for certain laboratory tests (sputum or stool cultures), respectively. Also, because many of the category A high-threat diseases are zoonoses (primarily infecting 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.

Potential Epidemiological Clues to an Unnatural Event

It is not possible to determine the objectives of a bioterrorism perpetrator in advance, whether the intent is to kill, incapacitate, or obtain visibility; or

how a biological agent may be 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 an unnatural event has occurred. The following compilation is an illustrative list; however, additional clues may be found elsewhere.^{8,9}

Clue 1: A highly unusual event with large numbers of casualties. Although the mention of BW or BT may elicit images of massive casualties, this may not actually occur with a real BW/BT event. Numerous examples of naturally spread illness have caused massive 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. If the illness is normally sensitive to certain antibiotics but displays resistance, then resistance may have been purposefully engineered. 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. If a case of a disease such as yellow fever, which is endemic to parts of South and Central 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 1999.¹⁰ 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 outbreak curve demonstrating the timeline of dates when patients developed illness. These timelines 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 propagates by person-to-person spread. It is thought that with an intentional BT event, a point source outbreak curve would be seen¹¹ in which individuals would be 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 3-2. The epidemic curve might be slightly compressed because infected individuals were exposed more closely in time (ie, within seconds to minutes of each other) from an aerosol release, compared with individuals becoming ill after eating a common food over a period of minutes to hours. The inoculum may also be greater than what is typically seen with natural spread, thus yielding a shorter incubation than expected.

Clue 5: Multiple epidemics. If a perpetrator can obtain and release a single agent, why could multiple perpetrators not do so with a single agent 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 combined, and would thus cause serial outbreaks of different diseases in the same population.

Clue 6: Lower attack rates in protected individuals. This clue is especially important to military personnel. If certain military units wore military-oriented protective posture (MOPP) gear or respiratory protection

(such as high-efficiency particulate air [HEPA]-filtered masks), or stayed in a HEPA-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 coal mines to detect the presence of noxious gases is one example. Because many biological agents that could be used for BW/BT are zoonoses, a local animal die-off may indicate a biological agent release that might also infect humans. This phenomenon was observed during the 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.^{12,13}

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. When Sin Nombre virus initially appeared in the desert southwest of the United States,¹⁴ environmental factors increased food sources and caused the field mouse (*Peromyscus maniculatus*) population to surge. The proliferating field mice encroached upon human habitats. The virus spread among the mice, causing a persistent infection and subsequent excretion in their urine.¹⁵ Humans close to the mice became infected. If human disease precedes animal disease or human and animal disease is simultaneous, then unnatural spread should be considered.

Clue 9: Unusual disease manifestation. Over 95% of worldwide anthrax cases are cutaneous illness. Therefore, a single case of inhalational anthrax may likely be an unnatural event. This logic may be applied to case reports of a disease such as plague, where the majority of naturally occurring cases are the bubonic, and not the pneumonic form. Any inhalational anthrax case may be caused by BW/BT unless proven otherwise. Perhaps the only exception would be an inhalational anthrax case in a woolen mill worker.

Clue 10: Downwind plume pattern. The geographic locations where cases occur can be charted on a geographic grid or map. If the reported cases are found to be clustered in a downwind pattern, 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 by 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

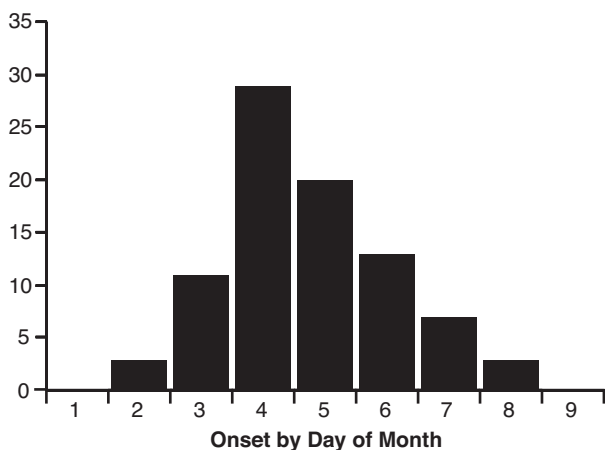


Fig. 3-2. Typical point source outbreak epidemic curve

leaves a signature. The signature could be a letter filled with anthrax spores,¹⁷ a spray device, or another vehicle for agent spread. It would then be useful to compare samples from such a device with the clinical samples obtained from victims to verify that they are the same organism.

Outbreak Investigation

It is important to understand the basic goals of an outbreak investigation, as seen in Exhibit 3-1. Any outbreak 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 quickly, 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.

With notification of any outbreak, whether natural or human-caused, there are standard steps to follow in an outbreak investigation (Exhibit 3-2), although these steps may not always occur in order.¹⁸ The first step is preparation, which involves having the necessary response elements (personnel, equipment, laboratory capabilities) ready, and establishing communications in advance with partners 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 broad case definition at first and avoid excluding any

EXHIBIT 3-1

GOALS OF AN OUTBREAK INVESTIGATION

- Find the source of disease
- Rapidly identify cases
- Prevent additional cases

EXHIBIT 3-2

TEN STEPS IN AN OUTBREAK INVESTIGATION

1. Prepare for fieldwork.
2. Verify the diagnosis. Determine an outbreak exists.
3. Define the outbreak and seek a diagnosis.
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.

potential cases too early. However, a definition should use clinical features that are objectively measured whenever possible, such as temperature exceeding 101.5°F, rash, bloody vomitus, or diarrhea. The case definition enables the investigator to count cases and compare exposures between cases and noncases. To obtain symptom information, it may not be sufficient to look at healthcare facilities only, but it will likely also be 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 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 3-2) 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 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

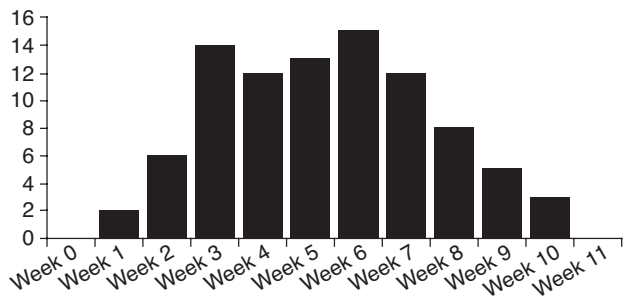


Fig. 3-3. Typical continuous common source outbreak epidemic curve

(Figure 3-3). With time and additional cases, the successive waves of illness may overlap with each other. 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 3-4]) than is seen with a point source of infection.

The fifth step is to develop exposure data with respect to person, place, and time. 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 start with individuals with and without disease and compare their potential exposures or risk factors for disease.

The sixth step is to implement control measures and continuously evaluate them. Control measures should be implemented as soon as possible. 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, it is useful to develop a hypothesis of 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. 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 there are some outliers who 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,

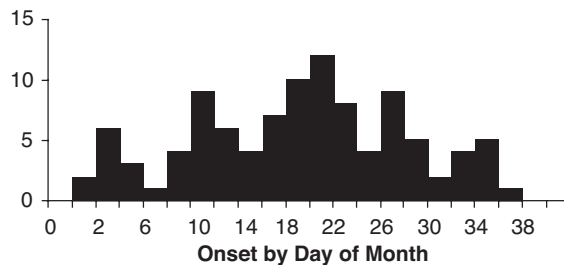


Fig. 3-4. Typical propagated (secondary transmission) outbreak epidemic curve

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 (step 10) through the media or medical literature, depending on the urgency of notification of 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 they can possibly collect, which may interfere with the investigation.¹⁹

As stated earlier, these different 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 spread of disease or the casualty toll is the ultimate test of whether the original hypothesis was correct.

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 should handle 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 be involved as early as possible in any suspected case as partners with public health officials in the investigation.²⁰

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 obtained in the investigation will be admissible in court. Public health authorities would need to use chain of custody for

environmental and clinical samples obtained during their investigation of a BT event. Environmental and biological samples can be crucial in determining whether a release has occurred (see the case study in this chapter about the release of anthrax 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 the safety of their personnel if there is a potential exposure risk during the investigation. Public health authorities conducting a field investigation should have personal protective equipment and be trained in its proper use, and have access to occupational health should pre- or postexposure prophylaxis be 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 have been considered as 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. As of the summer of 2007, the perpetrator of the anthrax mailings during the fall of 2001 had still not been apprehended by law enforcement authorities. Public health authorities initially considered the first inhalational anthrax death that occurred in this outbreak to have been naturally occurring. 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. 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 biological sabotage program was directed by the German army general staff and implemented 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.²¹ One 1916 German plan never carried out proposed to drop vats of plague cultures from Zeppelins over England.²²

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 then 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.²³ From the summer of 1915 through the fall of 1916, the cultures were used on horses and mules in holding pens in the 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.²⁴

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. A claim of effect upon the 1917 British advance on Baghdad during the Mesopotamian campaign is dubious. However, disease observed among animals might have originated naturally or from stressful holding and shipment conditions. One writer suspected that nonviable cultures may have originated from Tony's Lab because of the lack of illness among the saboteurs.²² However, using rubber gloves may have protected the plotters from acquiring cutaneous anthrax or glanders from the bacterial cultures.

If a similar incident occurred now, would current biological detection capabilities alert health officials? Glanders produces disease in horses, mules, and donkeys and is poorly transmitted directly to humans. The examining clinician should be suspicious when seeing persons exhibiting this disease without previous exposure to these animal vectors.

Few syndromic surveillance systems incorporate comprehensive veterinary surveillance. This is an important disease detection vulnerability because many of the BW agents (ie, *B anthracis*, *Brucella suis*, *B mallei*, *B pseudomallei*, *Coxiella burnetii*, *Francisella tularensis*, *Yersinia pestis*, encephalitis, and hemorrhagic fever viruses) can cause zoonotic illness. Furthermore, US industrial agricultural practices are vulnerable to the threat of antianimal agents.^{25,26} Few geographic areas have an established infrastructure that permits accurate and comprehensive animal disease reporting. 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 Network²⁷ and the Centers for Epidemiology and Animal Health,²⁸ both part of the US Department of Agriculture (USDA).

Depending on exposures and timing, a purposeful use of anthrax (*B anthracis*) or glanders (*B mallei*), such as the occurrence in 1915–1916, would likely be detected initially by hospital emergency department clinicians or physicians in private practice through their examination of affected persons, or by veterinarians inspecting animals for transport. If such an incident with large numbers of glanders or anthrax cases in animals about to be shipped overseas occurred now, detection might occur through the USDA Animal and Plant Health Inspection Service's inspection or record-keeping processes. Case-specific information for human cases would be reported to state health authorities, and ultimately the Centers for Disease Control and Prevention (CDC) would be notified.

Disease outbreak information exchange between federal partners such as CDC and USDA may eventually lead to a "one medicine approach" linking human and animal health reporting. A viable hospital emergency department syndromic surveillance network monitored by state health authorities could detect a cluster of patients with similar etiologies indicating anthrax. Law enforcement authorities might also interview sentinel cases from a suspect outbreak to investigate whether they could be outbreak perpetrators who had inadvertently become infected.

Lessons Learned: Veterinarians familiar with glanders

or anthrax in livestock and USDA select agricultural agents should report these diseases to state health and federal authorities as possible indicators of BT. Until recently, glanders had not occurred in the United States since 1945, when it was reported in military laboratory workers.²⁹ In 2000, 55 years later, a Maryland laboratory worker contracted glanders, demonstrating the continuing potential for risk of occupational exposure to this disease in biodefense laboratory workers,³⁰ as well as the paramount importance of adhering to biosafety level 3 standards. Endemic anthrax also occasionally occurs in the United States, along with zoonotic³¹ or laboratory transmission.^{32,33}

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.^{34,35} 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.³⁶

At the time (and now), public health authorities would not consider a foodborne salmonellosis outbreak initially as having been caused purposefully. It has been estimated that 1.4 million salmonellosis infections occur annually in the United States, resulting in 15,000 hospitalizations and 400 deaths.³⁷ Therefore, the index of suspicion for an intentional *Salmonella* outbreak was—and remains today—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 of the ill persons.³⁸

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.²¹ 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 *S typhi* (the causative agent of typhoid fever) and the human immunodeficiency virus, the Rajneeshees legally obtained cultures of *S typhimurium* (ATCC 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. Then the cult 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.³⁸

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.³⁸ 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. Employees of restaurants 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 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 distributors and suppliers of foods 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 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.³⁹ 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.^{21,38} 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.³⁸

Intentional contamination of the salad bars is consistent with the retrospective epidemiology.³⁸ 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.²¹

Case Review of 1984 Salmonellosis Outbreak

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

Potential Epidemiological Clues: 1, 4, 5, 11

Review: Only one commune member admitted to contamination of a salad dressing with a bacterial culture, and it is unknown what other food items the other perpetrators contaminated. Public health authorities found no statistical association with any single food item.²¹ 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 Rajneshees more than a year after the outbreak occurred. Public health and law enforcement authorities lacked cooperative protocols in 1984, yet the public health and law enforcement teams in Oregon worked well together, as demonstrated by a public health laboratory official accompanying the FBI investigation. This official noticed the *S typhimurium* culture, which may have gone unnoticed by the FBI. An outbreak of this magnitude would today initiate a joint inquiry and investigation by public health and law enforcement, increasing chances that the outbreak cause would be identified in a more timely manner.

Lessons Learned: These events illustrate the need to have joint public health and law enforcement investigations and mutual cooperation. Additionally, this outbreak shows the importance of the mode of disease spread in discerning whether it occurred naturally. An unlikely vehicle may be responsible for a deliberate foodborne disease outbreak. Although not occurring in this case, when different locations are involved, there could be a central supplier of a contaminated product shipped to all the locations.

Anthrax—Tokyo, Japan, 1995

The notorious sarin (a chemical nerve agent) attacks in a Tokyo suburb, Kameido, in 1994 and 1995, culminated with a sarin release in the Tokyo subway system.^{40,41} Less well known is that before their efforts with chemical weapons the apocalyptic cult Aum Shinrikyo appears to have first invested efforts into the production of biological agents and had tried to use them.²¹

Shoko Asahara, a charismatic guru, built the Aum Shinrikyo cult into a membership of 10,000 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 investigated the use of *C burnetii* (the bacteria 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 release 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.⁴² 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.⁴² It is not known whether a similar retrospective examination of anthrax-caused animal deaths was or could have been performed.

Case Review of 1995 Anthrax Releases

Biological Agents: *B anthracis*, gram-positive bacillus

Potential Epidemiological Clues: 11

Review: None of the biological attacks carried out by the Aum Shinrikyo cult were successful. In contrast, there were 12 deaths and about 1,000 hospitalizations from the sarin releases by the Aum Shinrikyo.⁴⁰ Technical errors in either the biological agent production or dissemination rendered the attacks harmless. The anthrax strain that the cult was using was likely a harmless strain used in animal vaccines.

In 2001 specimens from the exterior of the Tokyo building where the cult released anthrax spores were cultured to analyze the strain's genetic material. 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.⁴²

Even if the strain used was pathogenic, the concentration of spores in the liquid suspension is significantly less (10^4 bacteria/mL) than that considered optimal for a biological weapon (10^9 – 10^{10} bacteria/mL). The viscosity of the suspension was also problematic for successful aerosolization. Area residents described a gelatinous substance, suggesting poor dispersion. Also, the Aum Shinrikyo spray system's

effectiveness is doubtful; reports indicate it repeatedly broke down. Finally, 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.⁴² These experiences show that it is difficult to both create a pathogenic biological weapon and to use it. However, if the Aum Shinrikyo had obtained a different strain of *B anthracis*, the intended effects may have been more successful, which may have led the cult to use a biological agent in the Tokyo subway system. Its failures with biological agents led the group to use sarin, a chemical nerve agent.

Lessons Learned: 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. The Aum Shinrikyo knew that it could effectively use sarin from experience with an earlier release in the Matsumoto area of Tokyo in 1994.⁴⁰ If the cult had not failed to culture and develop biological agents, it may have used a combination biological and chemical weapon in 1995. Another lesson learned is the importance of environmental sample collection and proper storage. The emerging discipline of forensic molecular biology proved the occurrence of an anthrax release by analysis of archived samples 8 years after the incident.⁴³

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.²¹ *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 from 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 hospital emergency departments and released, four were hospitalized, but no deaths resulted.⁴⁴

During the subsequent epidemiological investigation, 45 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 there were no other cases in the community outside 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 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 non-outbreak *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 those ill persons, the uneaten muffin, and the laboratory's stock culture of *S dysenteriae* type 2. This specific pathogen was known to be uncommon. No research with this microorganism had been conducted at the hospital; therefore, laboratory technicians were not at risk of infection through laboratory error. No concurrent outbreaks of *S dysenteriae* type 2 were reported nationally at the time. Contamination of pastries 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. Therefore, health authorities did not order a food recall. When the epidemiological report was published,⁴⁴ 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.⁴⁵

Lessons Learned: A match of clinical, food, and laboratory isolates helped to prove an epidemiological link among them. In this case, only an individual with direct access to the laboratory culture could have committed this "biocrime," and one such person was eventually apprehended. In addition,

the epidemiological investigation was helped by the knowledge that only postproduction tampering of the baked goods could have resulted in their successful contamination.

Anthrax—USA, 2001

On October 4, 2001, an inhalational anthrax case was reported in a 63-year-old male in Florida.⁴⁶ Public health and government authorities initially misunderstood the nature of inhalational anthrax exposure and assumed that this individual had contracted the illness by outdoor hunting activities.⁴⁷ 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.⁴⁸ Investigators then realized that the exposures resulted from anthrax-containing letters placed in the mail. On October 15, a letter was received at Senate Majority Leader Tom Daschle's office that threatened an anthrax attack and also contained anthrax spores. The Hart Senate Office Building in Washington, DC, was subsequently closed.⁴⁹ 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.^{50,51} A 12th cutaneous anthrax case related to these mailings occurred in March 2002 in a Texas laboratory where anthrax samples had been processed.⁵²

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 because of these events. Massive public health and law enforcement investigations 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.⁵³ Public health surveillance to both detect previously unreported anthrax cases and to determine that no new cases were taking place severely strained public health capacity.^{54,55} Even states that did not have anthrax cases were inundated with requests from the public to test various pieces of mail and powder-containing articles. 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 laboratories⁵⁶ with national public health and military laboratories, served as a lead resource for both identifying and ruling out a potential biological attack.⁵⁷ Molecular subtyping of *B anthracis* strains played an important role in the differentiation and identification of anthrax. High-resolution molecular subtyping determined that the anthrax mail-related isolates were indis-

tinguishable and likely came from a single source.⁵⁸ Postal workers and others handling mail were shown to be at risk from the anthrax-containing letters⁵⁹ and contaminated postal machinery⁶⁰; therefore, environmental sampling,⁶¹ cleaning,⁶² and protective measures as well as antibiotic prophylaxis, were instituted by federal and state health officials.⁶³ Similar protective actions were taken after discovery of the anthrax spore-laden envelope opened in the Senate Office Building.⁴⁹ The continued monitoring of this population will provide invaluable information concerning anthrax exposures and the efficacy of prophylaxis.⁶⁴

Anthrax has been known to be an occupational hazard to industrial workers in the United States even before the causative organism *B anthracis* was isolated by Robert Koch in 1877.⁶⁵ As previously mentioned, German agents used anthrax as an agent for materiel sabotage in the United States during 1915 and 1916. As of the summer of 2007, the perpetrator of the anthrax mailings has still not been apprehended by law enforcement authorities. The anthrax mailings have irreversibly changed much of US society and greatly influenced the public's perception of vulnerability to an attack from a biological agent. In the month after public notification of confirmed cases, the CDC responded to over 11,000 phone calls.⁶⁶ A "crisis mode" prevailed at many state and local health departments, who also managed similar phone triage from the public. These agencies also received queries around the clock from healthcare providers presenting patient details and requesting clinical information to rule out anthrax, media queries, and reports of untold numbers of "white powder" incidents demanding instant identification of the substance.⁶⁷ In states where anthrax cases occurred, these demands were exacerbated by the need for anthrax exposure assessments for postal workers, patients, and workplace and home environments; distribution of pharmaceuticals; and exhaustive statewide prospective and retrospective anthrax-syndromic surveillance case review and reporting.⁶⁸ According to Casani, Matuszak, and Benjamin, government authorities sent conflicting messages on policies and priorities based on scientific knowledge that changed hourly, daily, and weekly.⁶⁷

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.⁶⁹ Disease detection and notification efforts, a cornerstone of BT preparedness, have changed dramatically since the incident with the implementation of automated laboratory reporting via the National Electronic Disease Surveillance System⁷⁰ and automated hospital syndromic surveillance reporting⁷¹ by public health agencies in many states and large cities. Continuing efforts to strengthen the public health workforce should help to better detect, respond, and manage a future BT crisis.⁷²

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. No one can anticipate how an initial case will present. The most important lesson learned in this outbreak is that 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 exposure.

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

After a terrorist plot to use ricin in England in January 2003,⁷³ this toxin was found in a South Carolina postal facility in October 2003.⁷⁴ Ricin was also discovered in the office of Senator Bill Frist at the Dirksen Senate Office Building in Washington, DC, on February 3, 2004.⁷⁵

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. All postal workers at the facility were then interviewed by state health authorities, 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.⁷⁴

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 potent 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 the presence of 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.⁷⁶

Any ricin threat should be investigated. Healthcare providers and public health officials must be vigilant for illness consistent with ricin exposure. However, in the above incidents, no cases resulted from exposure. It is likely that the material used in these incidents was not processed, purified, or dispersed in a manner that would cause human illness.

Accidental Release of Biological Agents

The following case studies document the events that transpired after what is understood to be the accidental release of two biological warfare agents, *B anthracis* and *Variola major*, in the former 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*.⁷⁷ These accounts place frightening emphasis on the dangers to innocent populations from purposeful biological weapon development.

Smallpox—Aralsk, Kazakhstan, 1971

An outbreak of smallpox occurred as a result of a field test at a Soviet biological weapons facility in 1971, largely unknown to the outside world until 2002.⁷⁸ Vozrozhdeniya (Renaissance) Island lies in the Aral Sea, and belongs jointly to the post-Soviet republics of Kazakhstan and Uzbekistan. In 1954 a biological weapons test site (Aralsk-7) was built on this island and on neighboring Komsomolskiy Island. The Soviet Ministry of Defense also established a field scientific research laboratory to conduct biological experiments on Renaissance Island.⁷⁹ BW agents tested here included *B anthracis*, *C burnetii*, *F tularensis*, *B suis*, *Rickettsia prowazekii*, *V major*, *Y pestis*, botulinum toxin, and Venezuelan equine encephalitis virus.⁸⁰

According to Soviet General Pyotr Burgasov, field testing of 400 g of smallpox caused this outbreak at Renaissance Island on July 30, 1971.⁷⁸ Ten persons contracted smallpox, and three unvaccinated individuals (a woman and two children) died from the hemorrhagic form of the disease. One crew member on the research ship the Lev Berg contracted smallpox as the ship passed within 9 miles of the island. This crew member became ill on August 6 with fever, headache, and myalgia. The ship then landed in the port city of Aralsk on August 11. The ill crew member returned to her home, and she developed a cough and temperature exceeding 102°F. Her physician prescribed antibiotics and aspirin. Although she was previously vaccinated for smallpox, a rash subsequently appeared on her back, face, and scalp; her fever subsided; and she recovered by August 15. On August 27 this patient's 9-year-old brother developed a rash and fever, his pediatrician prescribed tetracycline and aspirin, and he recovered.⁷⁹

During the following 3 weeks, eight additional cases of fever and rash occurred in Aralsk. Five adults ranging in age from 23 to 60, and three children (4 and 9 months old, and a 5-year-old) were diagnosed with smallpox both clinically and by laboratory testing. These children and the 23-year-old were previously unvaccinated. The two youngest children and the 23-year-old subsequently developed the hemorrhagic form of smallpox and died. The remaining individuals had previously been vaccinated, and all recovered after having an attenuated form of the disease.⁷⁹

A massive public health response to the smallpox cases in Aralsk ensued once the disease was recognized. In less than 2 weeks, approximately 50,000 residents of Aralsk were vaccinated. Household quarantine of potentially exposed individuals was enacted, and hundreds were isolated in a makeshift facility at the edge of the city. All traffic in and out of the city was stopped, and approximately 54,000 square feet of living space and 18 metric tons of household goods were decontaminated by health officials.⁷⁹

Case Review of 1971 Smallpox Outbreak

Biological Agents: *V major* virus

Potential Epidemiological Clues: 3, 4, 6, 10, 11

Review: The high ratio of hemorrhagic smallpox cases in this outbreak, combined with the rate of infectivity and the testimony of General Pyotr Burgasov (former Soviet vice-minister of health), has led to the understanding that an enhanced weaponized strain of smallpox virus was released from Aralsk-7 in 1971.⁷⁹ It may never be known whether the release was purposeful, but the Lev Berg inadvertently traveled into the plume of this bioweapons release, initiating the smallpox outbreak in Aralsk.

Lessons Learned: The Aralsk-7 BW facility had a history of association with mass deaths of fish, various regional plague outbreaks, a saiga antelope die-off, and individual cases of infectious disease among visitors to Renaissance Island.⁸⁰ These events present a timely warning for BW defense researchers working with biological agents that have the potential for infecting not only the laboratory workers, but also their family members and the surrounding community. Recent laboratory-acquired infections with tularemia,⁸¹ Sabia virus,⁸² and glanders⁸³ underscore the potential for risk of disease transmission in this manner. Considering that Lake and Francis reported six cases of laboratory-acquired tularemia in 1921,⁸⁴ this is not a new phenomenon. The epidemiological lesson learned is that when unusual BT-related illnesses occur, a laboratory accident or open air testing of a BW program may have occurred.

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 ingestion of *B anthracis* spores, although it occasionally occurs in the republics of the former Soviet Union.^{16,85} 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 This 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, for intensive care, and for autopsy. Anthrax illness was understood not to 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 put into place by authorities 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 ages 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.^{16,86}

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 about 14% more deaths would have occurred in Sverdlovsk in the absence of the public health intervention that included distribution of antibiotics and vaccination.⁸⁶ 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.⁸⁷ Ambulance personnel often made an initial case diagnosis of pneumonia.⁸⁸

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.⁸⁹

Former Soviet physicians released important information about anthrax prophylaxis and treatment, some of whom took tissue samples and records home at their own 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.⁸⁹ 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,⁹⁰ which belies the claim for a single-source, naturally occurring anthrax outbreak, and points toward the release of a BW anthrax formulation from Compound 19.

Lessons Learned: Retrospective pathology findings from victims, weather patterns, and geographic mapping can help to determine the outbreak source and also whether an outbreak was spread intentionally. Most importantly, the 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, they provided prophylactic antibiotics and vaccination and undertook protective environmental measures.

Studies of Natural Outbreaks for Potential Bioweapon Use

Although the following accounts are examples of naturally occurring outbreaks, they have components that raise suspicion that they were intentionally caused. Subsequent to the 1999 WNV outbreak in New York City, suggestions were made that Iraqi operatives covertly released a biological weapon. These allegations are based on documentation showing that CDC had provided Iraq with various biological agents from 1984 through 1993, including *Y pestis*, dengue and WNV,⁹¹ and the government of Iraq was known

to have had a covert biological weapons program.⁹² Similar allegations of the covert use of a biological weapon could have been made with the 2000 Martha's Vineyard, Massachusetts, tularemia outbreak and 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, five of whom had profound muscle weakness, and four of whom 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 3.⁹³

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-Barre syndrome, aseptic meningitis, or presence of the clinical syndrome as identified in earlier cases.⁹³ Before and during this outbreak, an observed increase in bird deaths (especially crows) was noted in New York City.¹² 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.⁹⁴ 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-diethyl-meta-toluamide (DEET)-based mosquito repellent 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.⁹³ A seroprevalence survey later determined that approximately 100 asymptomatic infections and 30 WNV fever cases occurred for each WNV encephalitis case in the New York City area.⁹⁵

Case Review of 1999 West Nile Virus Outbreak

Biological Agents: West Nile virus, a flavivirus

Potential Epidemiological Clues: 1, 2, 3, 7

Review: After this outbreak had occurred, author Richard Preston claimed in a magazine article that Cuba and Iraq had developed WNV as a bioweapon.⁹⁶ Although it may not be possible to disprove such a claim, it is even more difficult to substantiate. 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.⁹³ Limitations of serologic testing underscore the importance of isolation and identification of virus.⁹³ 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.⁹⁷ WNV from this part of the world occasionally causes epidemics in Europe that may be initiated by migrant birds.^{98,99} 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.⁹³

WNV is transmitted primarily by *Culex pipiens* mosquitoes,¹⁰⁰ which contributed to its spread in the United States after the 1999 outbreak.¹⁰¹ Therefore, nationwide public health mosquito surveillance was subsequently instituted. Genetic testing revealed that the virus was 99% identical to a virus isolated in 1999 from a goose in Israel.¹⁰² 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.¹⁰³ Before this outbreak, death was rarely associated with WNV infection.¹⁰⁴ In patients with WNV encephalitis, computer-assisted tomography often revealed preexisting lesions and chronic changes in brain tissue,¹⁰⁵ 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.¹⁰⁴ The incident is an example of a typical zoonotic disease epidemic pattern—a natural epidemic occurred first among birds, followed by disease in humans. Once WNV became established within the indigenous North American mosquito vectors, it spread and

has become endemic to the continent. 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), are not uncommon. Regardless of origin, outbreak investigation steps remain the same, as does the need for a robust public health surveillance, investigation, and response system.

Tularemia, Martha's Vineyard, Massachusetts, 2000

During the summer of 2000, an outbreak of primary pneumonic tularemia occurred on Martha's Vineyard, Massachusetts.¹⁰⁶ 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, 2 had ulceroglandular disease, and 2 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.

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 who 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.

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.

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 1990 and 2000, an average of 124 cases per year was reported.¹⁰⁷ Over 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 years, and incidence was greatest among American Indians and Alaska natives.¹⁰⁷

The only other previously reported pneumonic tularemia outbreak in the United States had occurred on Martha's Vineyard during the summer of 1978.¹⁰⁶ During a single week (July 30–August 6) seven persons stayed in a vacation cottage. By August 12, six of them 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,¹⁰⁶ and pneumonic tularemia was first reported in Massachusetts in 1947.¹⁰⁸ Classic research on human tularemia rates showed that very high rabbit populations increase the tularemia hazard.¹⁰⁹ Hospital clinicians on Martha's Vineyard initially detected this outbreak and recognized tularemia-caused pneumonic summer illness,¹¹⁰ in part based on the experiences with the previous outbreak.¹⁰⁶

In the 2000 outbreak of tularemia, Feldman et al proposed that on Martha's Vineyard, *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.¹¹¹ 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.¹¹²

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.¹¹⁰ There may also be disease transmission mechanisms (in this example, grass cutting) that are unknown or poorly understood.

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 1974.¹¹³ By April 2000, 250 suspected cases had been identified and spread nationwide,

but with most cases in the western area where ethnic Albanians resided.¹¹⁴

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, typhus, plague, cholera, dysentery, typhoid fever, and smallpox have long been observed in war-torn regions.¹¹⁵ Among early examples is the plague of Athens that arose during the second year of the Peloponnesian War, as described by Thucydides.¹¹⁶ Speculation may arise that these epidemics were purposefully caused. Many biological agents are zoonotic pathogens,¹¹³ including tularemia, a category A BW pathogen. Purposeful use of this pathogen merits consideration when such an outbreak occurs with a potential BW pathogen.¹¹⁷ 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 BW by Serbian forces.¹¹³

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.¹¹⁸ To further complicate matters, a 1998 report documented that type A tularemia had been introduced into arthropod populations in the nearby Slovak Republic.¹¹⁹

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.¹²⁰ 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.¹¹³

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, over 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. The protective value of eating fresh vegetables may be related to a minimal storage life and lessened opportunity for contamination.

Purposeful use of tularemia was considered. 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. Upon return to their villages, refugees discovered bombed and ransacked homes, unprotected food storage areas, unharvested crops, damaged wells, and a rodent population explosion. Both ignorance of infection and lack of hygienic measures contributed to a foodborne infection in the population.¹¹³ These factors likely resulted in conditions favorable for epizootic tularemia spread in rodents and widespread environmental contamination with *F tularensis* because this organism 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.¹¹³

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 over 300 cases of oropharyngeal tularemia.¹²¹ Historically, war in Europe caused tularemia outbreaks. During World War II, an outbreak of over 100,000 cases of tularemia occurred in the Soviet Union,¹²² and outbreaks with hundreds of cases following the war occurred in Austria and France.¹²¹

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 investigation must be conducted to conclude or disprove that a BW event has occurred.

EPIDEMIOLOGICAL ASSESSMENT TOOL

It is especially useful for public health authorities to quickly determine whether an infectious disease outbreak is intentional or naturally occurring. Grunow and Finke developed an epidemiological assessment tool to rule out biological agent use during infectious disease outbreaks. This assessment tool's relevance was demonstrated by analysis of the 1999 through 2000 Kosovo tularemia outbreak.¹¹³ 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 or proof of the release of an agent as a biological weapon. Neither of these conclusive proofs occurred in Kosovo. With conclusive criteria, additional confirmatory information is unnecessary.¹¹³

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? Natural occurrence of tularemia in Kosovo, even in the absence of a previous outbreak, needed to be considered.
- **Biothreat.** Does a biological threat exist by virtue of a group having a BW agent and credibly threatening to use it? In Kosovo there was no evidence of a biological threat.
- **Special aspects.** Is there plausible evidence of purposeful manipulation of a pathogen? In Kosovo, bacterial cultures were not created because of a lack of resources and fear of laboratory transmission, so purposeful manipulation could not be determined.
- **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. A 25-year absence of reported tularemia did not eliminate the potential occurrence of an epidemic.
- **Environmental concentration.** Is there a high environmental concentration of the pathogen? The almost exclusive occurrence of oropharyngeal tularemia in Kosovo likely indicated ingestion of a high number of bacteria that could occur through food or water contamination. *F tularensis* was not found in drinking water and soil, but was discovered in rodent vectors.
- **Epidemic intensity.** Is the course of illness relative to disease intensity and spread in the population expected in naturally occurring illness? Because tularemia was absent in Kosovo before the epidemic, the 2000 outbreak was considered to be unusually intensive.
- **Transmission mode.** Was the path of disease transmission considered naturally occurring? A naturally occurring epidemic in itself does not rule out the purposeful use of a BW agent.
- **Time.** Was the calendar time of the epidemic unusual? The Kosovo epidemic began in October 1999, peaked in January 2000, and ended in May, which is a typical seasonal pattern for a naturally occurring European tularemia epidemic.
- **Unusually rapid spread.** Was the spread of the epidemic unusually rapid? The Kosovo epidemic was unusual in that within a brief

time period tularemia appeared throughout almost the entire Albanian territory.

- **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. In the Kosovo epidemic, the Serbian population was not found to have been purposefully spared from a BW attack, and poor hygiene and living conditions probably facilitated the disease spread in the ethnic Albanian population.
- **Clinical.** Were the clinical manifestations of the disease to be expected? During the Kosovo outbreak, clinical diagnosis was made more difficult by the simultaneous appearance of mumps and tuberculosis in the population.¹¹³

The Grunow-Finke epidemiological assessment procedure (Table 3-1) 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 upon 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 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 some time 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 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

TABLE 3-1
EPIDEMIOLOGICAL ASSESSMENT AND EVALUATION OF CASE STUDY OUTBREAKS

Nonconclusive Criteria	Assessment (possible points)	Weighting Factor	Maximum No. of Points	1915					
				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	2000				
	1999 WNV NYC	1999 Tularemia Kosovo	2000 Tularemia Martha's Vineyard	2001 Anthrax USA	2003 Ricin USA
Biorisk	6	2	0	6	6
Biothreat	6	3	0	6	9
Special aspects	0	0	0	9	0
Geographic distribution	3	3	3	3	3
Environmental concentration	4	4	4	6	6
Epidemic intensity	3	3	3	3	0
Transmission mode	2	2	6	6	0
Time	1	0	3	3	0
Unusually rapid spread	3	1	3	3	0
Population limitation	0	0	2	3	0
Clinical	1	1	3	3	0
Score	29	19	27	51	24

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

exercise underscores the challenges facing epidemiologists in determining whether a BT/BW event has occurred, unless 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. Disease reporting can be initiated throughout the illness exposure and the incubation period; the healthcare provider presentation; and the initial diagnoses, labo-

ratory testing, and patient hospital visit. Clinicians, laboratories, hospitals, ancillary healthcare professionals, veterinarians, medical examiners, morticians, and others may be partners in reporting the disease to public health authorities.

If a medical surveillance system first detects a biological attack, there may be a significant number of cases, and the available time to prevent further illness is short or already over. The point of release is the earliest detection point of a biological event. Some disease could be prevented at the point of release through publicized avoidance of the area, prophylactic medication use or vaccination of those exposed, and 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 materials¹²³ and help determine the presence and geographic extent of a biological release to focus emergency public health response and consequence management.

Although deployed sensors may detect an agent's release, the infinite number of venues and limited resources to deploy sensors and analyze air samples minimize the chances that an agent release will occur within range of an environmental monitor. In this case, the earliest opportunity to detect an attack will be 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 with the category A biological agents, one case will launch an investigation, as seen during the 2001 anthrax attacks.⁵⁰ 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, an overwhelming number of people may 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 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 is 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.¹²⁴ 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. This data can be from new or existing sources.¹²⁵ 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.¹²⁶

Numerous regional and national syndromic surveillance systems have recently 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) and other information (eg, pharmacy sales, absenteeism) 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, 2001,¹²⁷⁻¹²⁹ or during large gatherings for sports or other events¹³⁰; or they can be sustained systems for continuous surveillance.^{131,132} 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 and absenteeism. 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 the CDC.

In the US Department of Defense, the Electronic Surveillance System for the Early Notification of Community-based Epidemics (ESSENCE) uses outpatient diagnostic *International Classification of Diseases, Ninth Revision* codes and pharmacy prescriptions to track disease groups in military beneficiaries. The system has been expanded in some locations to include civilian data such as hospital emergency department chief complaints, over-the-counter pharmacy sales, outpatient billing codes, school absenteeism, and laboratory test orders.^{133,134} 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.¹³⁵ This system is available for all permanent US military treatment facilities worldwide and also for some deployed forces in the Middle East. Civilian versions of ESSENCE are also deployed to select cities through the Department of Homeland Security's BioWatch program.

Public health departments such as the New York City Department of Hygiene and Mental Health have also developed surveillance systems based on data already collected for other purposes. New York City uses coded 911 calls, hospital emergency department chief complaints, retail pharmacy sales, and work absenteeism data.¹³⁶ The department has detected communitywide increases in gastrointestinal and respiratory illnesses and reassured the public during high-profile public events that no evidence of outbreaks had been found.¹³⁷

The University of Pittsburgh's Realtime Outbreak Detection System (RODS) uses the National Retail Data Monitor and hospital emergency department chief complaints to detect and track disease outbreaks.^{138,139} Nearly 20,000 retail pharmacy, grocery, and mass merchandise stores participate in the National Retail Data Monitor, which monitors sales of over-the-counter healthcare products.¹⁴⁰ In addition, to integrate health data for earlier outbreak detection program, the RODS laboratory provides assistance to some health departments that participate in the BioWatch biosensor.¹⁴¹ As of 2004, RODS has been deployed in 10 US sites and one international site.¹⁴²

CDC has developed the BioSense program using national data sources such as the Department of Defense and Department of Veterans Affairs outpatient diagnostic codes, as well as laboratory test orders from a commercial vendor, to track disease patterns nationwide. The information is provided in a web-based format to health departments.¹⁴³ Algorithms are run on the data and send out an alert when levels of outpatient 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. BioSense is one part of the Public Health Information Network, an organization whose 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.⁷¹ The warning may not be early enough or effective countermeasures may not be available. In addition, although nonspecific data such as absenteeism 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, 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 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.

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, and robust disease surveillance systems will improve our ability to respond to any future BT or BW event.

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Chapter 4

ANTHRAX

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INTRODUCTION AND HISTORY

THE ORGANISM

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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.¹⁴ 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.^{5,6} 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.^{7,8}

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.^{9,10} Anthrax also was the first disease for which an effective live bacterial vaccine was developed; Louis Pasteur developed that vaccine in 1881.¹¹ Additionally, anthrax represents the first described occupational respiratory infectious disease. During the latter half of the 19th century, inhalational anthrax,¹² a previously unrecognized form, occurred among woolsorters 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.¹³

The military has long been concerned about anthrax 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. Anthrax 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. 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 may have been 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.¹⁴ Cases were also reported in animals located more than 50 km from the site.^{15,16} Polymerase chain reaction examination of tissue samples collected from 11 of the

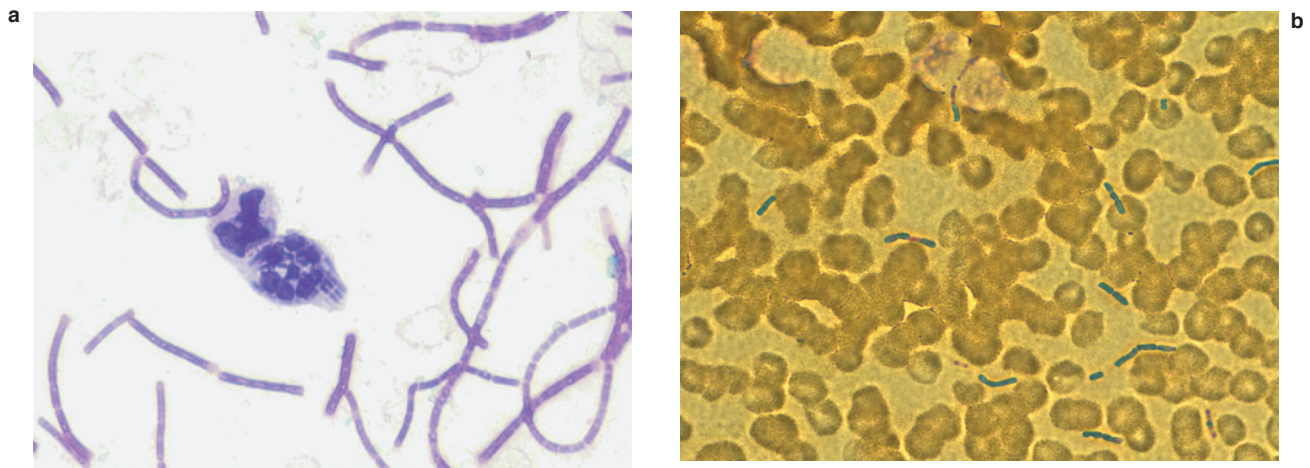


Fig. 4-1. (a) Gram stain of a blood smear from an infected guinea pig demonstrating intracellular bacilli chains within a polymorphonuclear leukocyte. (b) Gram stain of peripheral blood smear from a nonhuman primate infected with *Bacillus anthracis*, Ames strain.

Photograph: Courtesy of Susan Welkos, PhD, Division of Bacteriology, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

Photograph: Courtesy of John Ezzell, PhD, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

victims demonstrated that virulent *B anthracis* DNA was present in all these patients, and at least five different strains of virulent anthrax were detected based on variable number tandem repeat analysis.¹⁷

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 the press and of Congress, was mailed through the US Postal Service.¹⁸⁻²⁰ According to the Centers for Disease Control and Prevention, 22 people contracted anthrax from the letters.^{18,21-25} 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.²⁶⁻²⁹ This incident profoundly affected the law enforcement, scientific, and medical communities within the United States. As a result of the attacks, there has been increased governmental and public awareness of the threat posed by *B anthracis* and other pathogens, particularly those with a potential for aerosol-mediated infection.³⁰⁻⁴³ The amount of funding budgeted to prepare and protect the nation from a bioterror attack has rapidly increased since 2001, and a significant amount of this funding has supported anthrax studies. Some of the new anthrax studies have focused on improved sample collection,

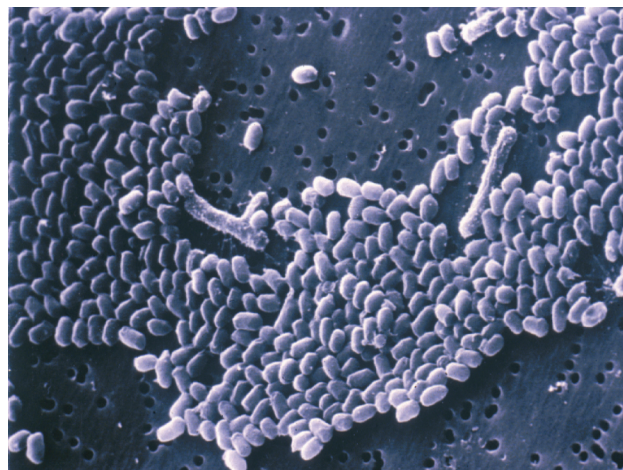


Fig. 4-2. Scanning electron micrograph of a preparation of *Bacillus anthracis* spores. Two elongated bacilli are also presented among the oval-shaped spores. Original magnification $\times 2620$.

Photograph: Courtesy of John Ezzell, PhD, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

rapid detection/diagnosis, decontamination, and microbial forensics. Because of the ongoing terrorism threat, there has been a particular sense of urgency regarding the development and improvement of medical countermeasures, such as therapeutics, vaccines, diagnostics, and devices.

THE ORGANISM

B anthracis is a large, gram-positive, spore-forming, nonmotile bacillus ($1-1.5 \mu\text{m} \times 3-10 \mu\text{m}$) that is closely related to *B cereus* and *B 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 and carbon dioxide and in tissue in vivo. In tissue, the encapsulated bacteria occur singly or in chains of two or three bacilli (Figure 4-1). The organism does not form spores in living tissue; sporulation occurs only after the infected body has been opened

and exposed to oxygen. The spores, which cause no swelling of the bacilli, are oval and occur centrally or paracentrally (Figure 4-2). The spores are very resistant and 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.^{44,45} Additional confirmatory tests to identify toxin and capsule genes by polymerase chain reaction, developed as research tools, have been incorporated into the Laboratory Response Network established by the Centers for Disease Control and Prevention.⁴⁶⁻⁴⁹

EPIDEMIOLOGY

Anthrax, 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.^{50,51} The form of the organism in infected animals is the bacillus. Sporulation occurs only when the organism in the carcass is exposed to air.

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.⁵² 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.^{51,53}

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 and ingesting contaminated meat. Cases associated with industrial exposure, rarely seen now, 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. 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.⁵⁴

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.⁵⁵ 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.⁵⁶⁻⁶⁰ In the 1996–1997 global anthrax report, there appeared to be a general decrease in anthrax cases worldwide; however, anthrax remains underdiagnosed and underreported.⁶¹ 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. 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.^{62,63} 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.⁶⁴ 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.⁶⁵ 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. Essentially all cases were cutaneous, including rare gastrointestinal disease cases and eight inhalational anthrax cases, although no autopsy confirmation was reported.⁶⁶

PATHOGENESIS

B anthracis possesses two protein exotoxins, known as the lethal toxin and the edema toxin; an antiphagocytic capsule; and other known and putative virulence factors.⁶⁷ The role of the capsule in pathogenesis was demonstrated in the early 1900s, when anthrax strains lacking a capsule were shown to be avirulent.⁶⁸ In more recent years, the genes encoding synthesis of the capsule were encoded on the 96-kilobase (kb) 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.⁶⁹ The capsule is composed of a polymer of poly-D-glutamic acid, which confers resistance to phagocytosis and may contribute to the resistance of anthrax to lysis by serum cationic proteins.⁷⁰ Capsule production is necessary for dissemination to the spleen in a murine inhalational anthrax model.⁷¹ Recently, the capsule has also been the focus of several efforts to develop new generation

anthrax vaccines.⁷²⁻⁷⁴ Evidence indicates that the capsule may enhance the protection afforded by PA-based vaccines against anthrax if opsonizing antibodies are produced.⁷⁴

Koch first suggested the importance of toxins in his initial studies on anthrax. In 1954 Smith and Keppie⁷⁵ 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.⁷⁶⁻⁷⁸ Advances in molecular biology in the past decade have produced a more complete understanding of the biochemical mechanisms of action of the toxins and have begun to provide a more definitive picture of their role in the pathogenesis of the disease.

Two protein exotoxins, known as the lethal toxin and edema toxin, are encoded on a 182-kb plasmid (pXO1), distinct from that coding for the capsule. In an

environment of increased bicarbonate, carbon dioxide, 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.^{67,79-82} 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.^{83,84}

Recently, a retrospective study identified an isolate of *B cereus* that carried a plasmid homologous to the anthrax toxin plasmid pXO1. This strain was obtained from a patient with symptoms similar to inhalational anthrax.⁸⁵ This finding led to considerable concern because “anthrax toxin” sequences are considered unique to *B anthracis*. Although a polyglutamate capsule was not produced, sequences encoding a polysaccharide capsule were present on a smaller plasmid. The possibility of false positives from toxin-based identification tests should be considered because many diagnostic schemes have focused on toxin genes and gene products. The virulence of this isolate has not yet been extensively studied, and the role of the lethal and edema toxins in the pathogenesis of this strain is unknown. Likewise, the incidence of such strains in nature is unclear. 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.⁸⁵ Other human cases of anthrax-like *B cereus* infections have been reported.^{86,87}

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 4-3). The B and A anthrax toxin components are synthesized from different genes and are secreted as noncovalently linked proteins. The anthrax toxins are unusual in that the B protein, PA, is shared by both toxins. Thus, the lethal toxin is composed of the PA₆₃ (MW 63,000 after cleavage from a MW 83,000 protein) heptamer combined with a second protein, which is known as the lethal factor (LF [MW 90,000]), and the edema toxin is composed of PA complexed with the edema factor (EF [MW 89,000]). Each of the 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^{69,88} or the PA gene alone⁸⁹ attenuates the organism. The lethal toxin appears to be more important for virulence in a mouse model than the edema toxin.⁹⁰ Crude toxin

preparations have been shown to impair neutrophil chemotaxis⁹¹ and phagocytosis.⁷⁰

The edema toxin causes edema when injected into the skin of experimental animals and is likely responsible for the marked edema often present at bacterial replication sites.^{92,93} 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.⁹⁴⁻⁹⁶ Edema toxin also impairs dendritic cell function and appears to act with lethal toxin to suppress the innate immune response.⁹⁷

The lethal toxin is a zinc metalloprotease that is lethal for experimental animals^{92,93,98} and is directly cytolytic for macrophages, causing release of the potentially toxic cytokines interleukin-1 and tumor

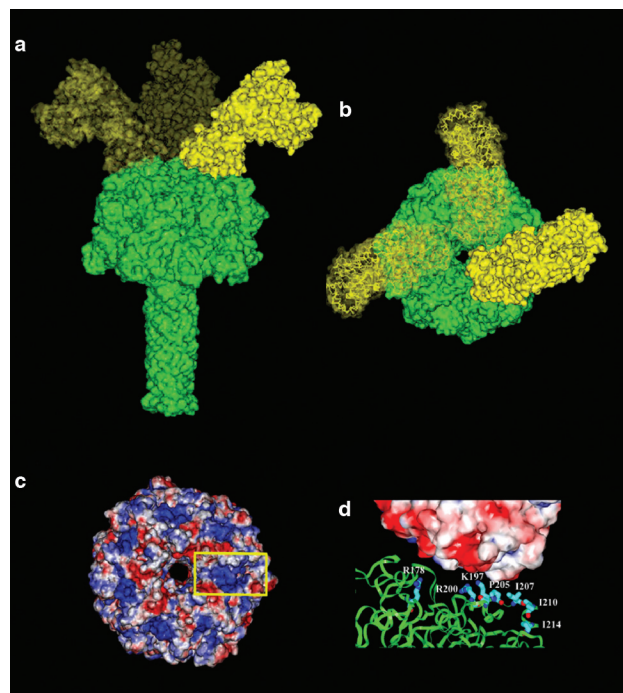


Fig. 4-3. Composition of anthrax lethal protein toxin. Molecular models of the protective antigen (PA)₆₃ heptamer and the PA₆₃ heptamer-lethal factor (LF) complex. (a, b) Side and top views of PA₆₃ heptamer (green) bound to three LF molecules (yellow). (c, d) The surface renderings are colored according to the negative (red) and positive (blue) electrostatic surface potential. (c) Top view of the PA₆₃ heptamer. The yellow box highlights the protomer-protomer interface and where LF binds to heptameric PA. (d) A hypothetical PA₆₃ heptamer-LF interface.

Photographs: Courtesy of Kelly Halverson, PhD, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

necrosis factor.⁹⁹ In in-vitro models, lethal toxin 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, lethal toxin interferes with the MAPK signaling pathways necessary for a multitude of normal cell functions.¹⁰⁰ In macrophage and dendritic cell models, lethal toxin leads to inhibition of proinflammatory cytokines, downregulation of costimulatory molecules, and ineffective T-cell priming.¹⁰⁰⁻¹⁰³ It also appears to promote apoptosis of endothelial cells lining the vascular system in vitro, leading to speculation that lethal toxin-induced barrier dysfunction leads to the vascular permeability changes accompanying systemic anthrax infection.¹⁰⁴ Effects on hormone receptors, including glucocorticoids, have also been reported. Although much of the information regarding lethal toxin activity has been obtained from animal-derived cell culture models, Fang et al recently reported that, in vitro, lethal toxin inhibits MAPK kinase dependent interleukin-2 production and proliferative responses in human CD4⁺ T cells.¹⁰⁵ The in-vivo targets for these toxins await confirmation; however, both lethal and edema toxins contribute significantly to suppression of the innate immune system.

Recent studies in cell culture models have given a clearer understanding of the molecular interactions of the toxin proteins.¹⁰⁰ PA first binds, most likely by a domain at its carboxy-terminus, to a specific cell receptor.¹⁰⁶⁻¹⁰⁸ Two proteins have been proposed as the PA receptor: (1) anthrax toxin receptor protein, ANTX1; and (2) capillary morphogenesis protein, CMG2.^{109,110} Although their natural ligands have not been identified, 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 (kd) fragment of PA on the cell surface.^{111,112} 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.^{101,113} Heptamerization stimulates endocytosis of PA (or PA-EF/LF complexes), which is then delivered to early endosomes.¹¹⁴ The mildly acidic pH of the endosome triggers membrane insertion of the heptameric PA into intraluminal vesicles.¹¹⁵ EF

and LF are translocated into the lumen of the vesicle and are thereby protected from lysosomal proteases.¹¹⁵ The toxins are then translocated via endosomal carrier vesicles to the cell cytosol, where they express their toxic activity.¹¹⁵

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.¹¹⁶ The proteolytic activation of PA necessary to form lethal or edema toxin may occur in interstitial fluid or serum rather than on the cell surface.¹¹⁶ The lethal or edema toxin may then bind to target cells and be internalized. This theory was recently bolstered by Panchal et al who demonstrated that purified LF complexed with the PA(110) heptamer cleaved both a synthetic peptide substrate and endogenous MAPK kinase substrates and killed susceptible macrophage cells.¹¹⁷ In addition, complexes of the heptameric PA(110)-LF found in the plasma of infected animals showed functional activity.¹¹⁷ Terminally, toxin is present in high concentrations in the blood, but the molecular mechanisms that cause death remain unknown.⁷⁶

Infection begins when the spores are inoculated through the skin or mucosa. Spores are ingested at the local site by macrophages, in which they germinate to the vegetative bacillus with production of capsule and toxins. At these sites, the bacteria proliferate and produce the edema and lethal toxins that impair host leukocyte function and lead to the distinctive pathological findings: edema, hemorrhage, tissue necrosis, and a relative lack of leukocytes. In inhalational anthrax, the spores are ingested by alveolar macrophages, which transport them to the regional tracheobronchial lymph nodes, where germination occurs.¹¹⁸ 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).¹¹⁹ The bacilli can then spread to the blood, leading to septicemia with seeding of other organs and frequently causing hemorrhagic meningitis. Death is the result of respiratory failure associated with pulmonary edema, overwhelming bacteremia, and often 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.^{120,121} 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.¹²² The vesicle, which may be 1 to 2 cm in diameter, ruptures, leaving a necrotic ulcer (Figure 4-4). Satellite vesicles may also be present. The lesion is usually painless, and varying degrees of edema may be present around it.¹²³ The edema may occasionally be massive, encompassing the entire face or limb, which is described by the term “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.^{124,125} Septicemia is rare, and with treatment mortality should be less than 1%.^{124,126-128} In addition, no age-related risk factor appears to be associated with cutaneous human anthrax.¹²⁹

Inhalational Anthrax

Inhalational anthrax begins after an incubation period of 1 to 6 days with nonspecific symptoms of malaise, fatigue, myalgia, and fever.¹³⁰⁻¹³³ 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 4-5). In the 2001 bioterrorist event, the pleural effusions were initially small but rapidly progressed and persisted despite effective antibiotic therapy.^{134,135} The effusions were predominantly serosanguineous and immunohistochemistry revealed the presence of *B anthracis* cell walls and capsule antigens. Effusion fluid from deceased patients who had received fewer than 55 hours of

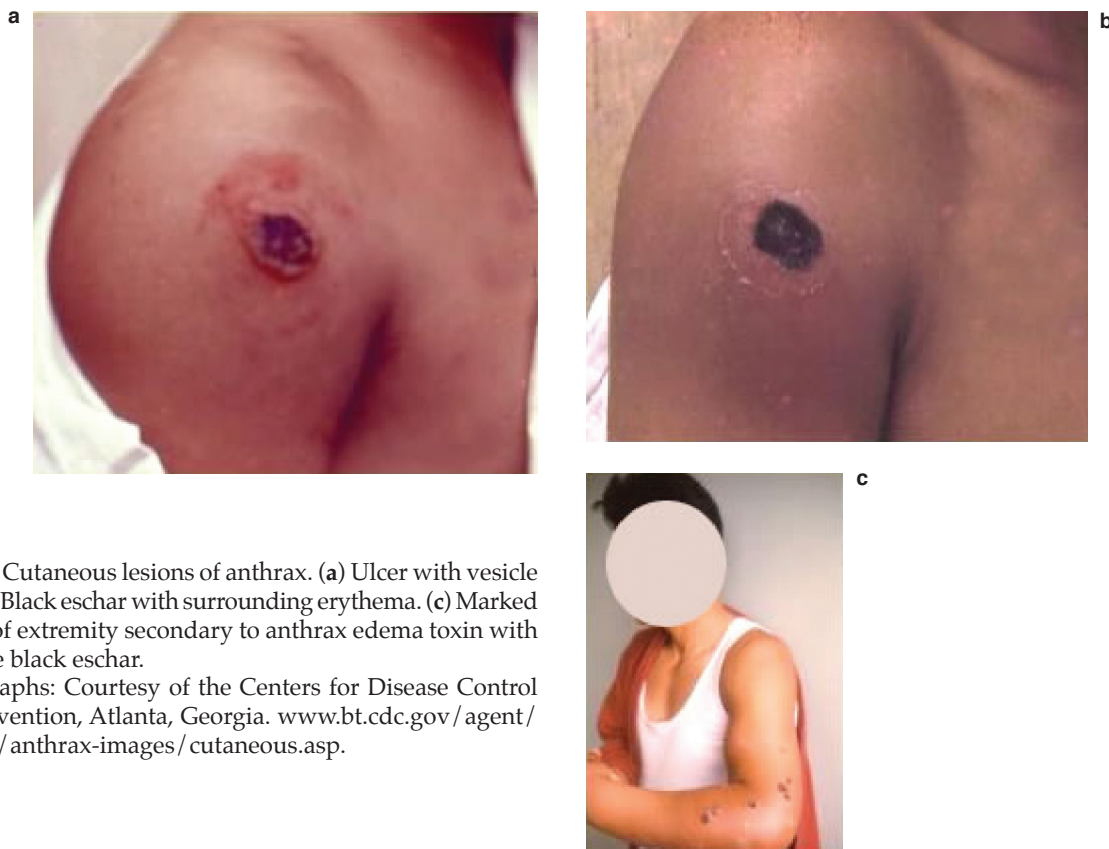


Fig. 4-4. Cutaneous lesions of anthrax. (a) Ulcer with vesicle ring. (b) Black eschar with surrounding erythema. (c) Marked edema of extremity secondary to anthrax edema toxin with multiple black eschar.

Photographs: Courtesy of the Centers for Disease Control and Prevention, Atlanta, Georgia. www.bt.cdc.gov/agent/anthrax/anthrax-images/cutaneous.asp.

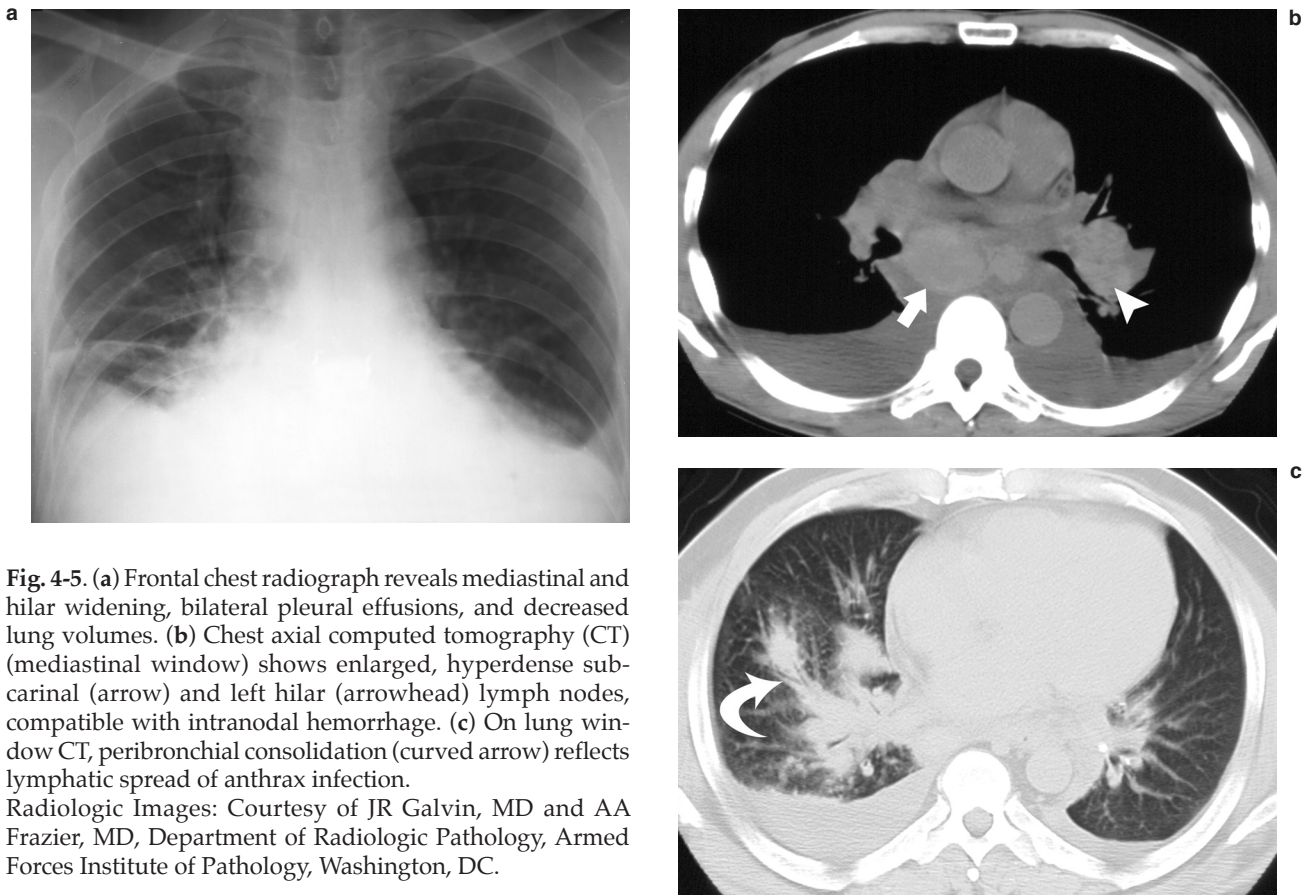


Fig. 4-5. (a) Frontal chest radiograph reveals mediastinal and hilar widening, bilateral pleural effusions, and decreased lung volumes. (b) Chest axial computed tomography (CT) (mediastinal window) shows enlarged, hyperdense subcarinal (arrow) and left hilar (arrowhead) lymph nodes, compatible with intranodal hemorrhage. (c) On lung window CT, peribronchovascular 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.

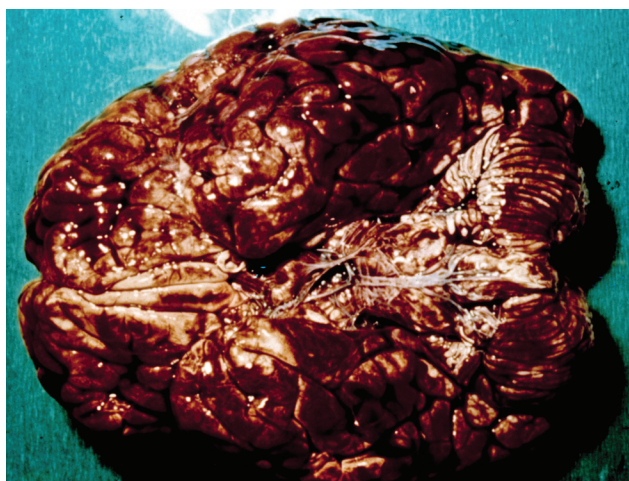


Fig. 4-6. Meningitis with subarachnoid hemorrhage in a man from Thailand who died 5 days after eating undercooked carabao (water buffalo).

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.

antibiotic therapy revealed the presence of bacilli.¹³⁶ Polymerase chain reaction analysis of the pleura fluid was also positive for *B anthracis* DNA.¹³⁷ Pneumonia has not been a consistent finding but can occur in some patients⁵ and may be attributed to intravascular edema and hyaline membrane formation.¹³⁶ 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.⁵² Meningitis is present in up to 50% of cases, and some patients may present with seizures. The onset of respiratory distress is followed 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%.^{134,135}

Oropharyngeal and Gastrointestinal Anthrax

Oropharyngeal and gastrointestinal anthrax result from ingesting infected meat that has not been sufficiently cooked.¹³⁸ 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.

Meningitis

Meningitis may occur after bacteremia as a complication of any of the other clinical forms of the disease.¹³⁹ 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 4-6).

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 4-7). 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 (see Figure 4-7 and Figure 4-8). Commercial polymerase chain reaction kits specific for the *B anthracis* pX01 and pX02 plasmids are also available to assist in identification of this organism. The differential diagnosis should include tularemia, staphylococcal or streptococcal disease, and orf (a viral disease of sheep and goats transmissible to humans).

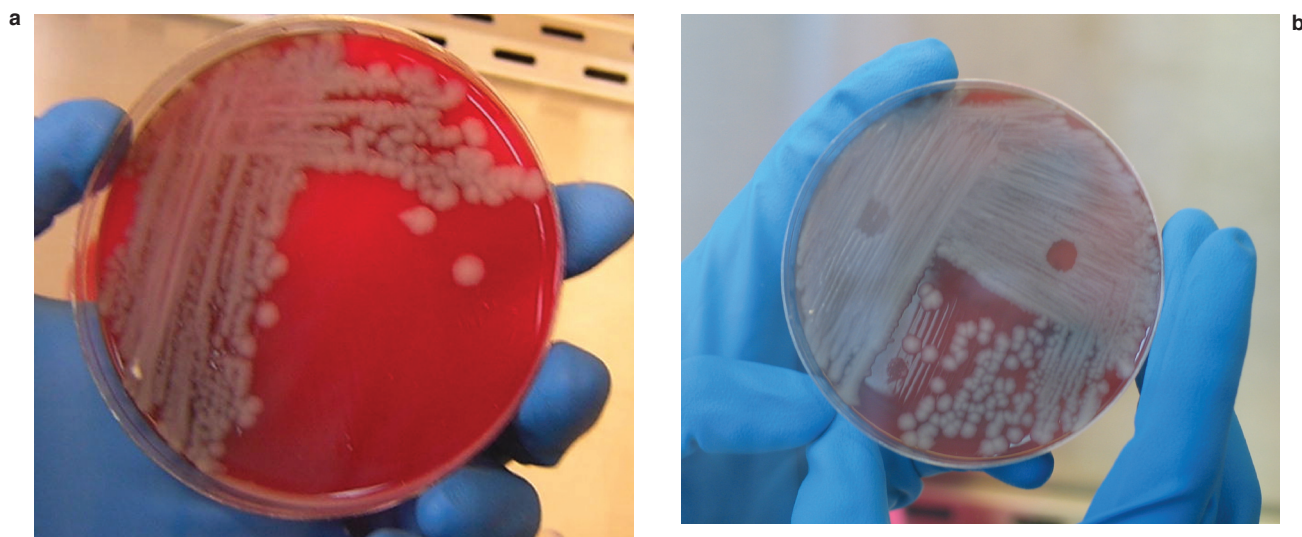


Fig. 4-7. (a) Isolated colonies of *Bacillus anthracis* on sheep blood agar plate. (b) Detection of *B anthracis* using specific gamma-phage mediated cell-lysis.

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.

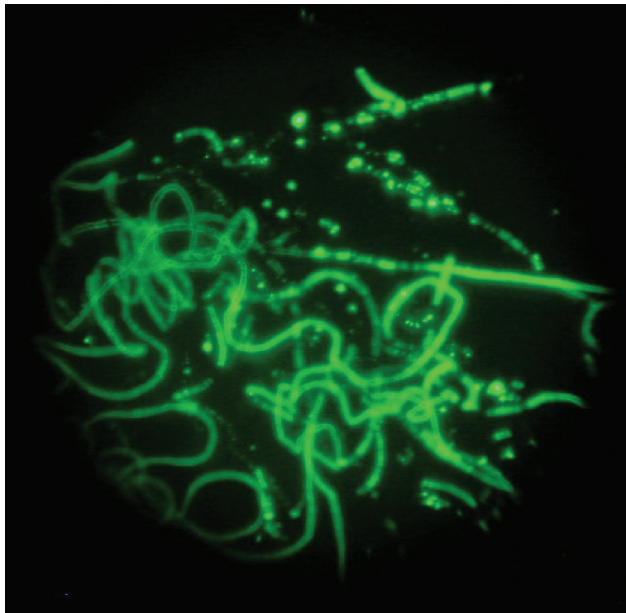


Fig. 4-8. Direct fluorescent antibody stain of *Bacillus anthracis* capsule.

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.

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 symptoms are nonspecific¹³¹⁻¹³³ 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, hemorrhagic pleural, and pericardial effusions.¹⁴⁰ During the later stages of the disease patients develop sudden fever, dyspnea, diaphoresis, cyanosis, hypotension, shock, and death.¹³¹ Blood culture should demonstrate growth in 6 to 24 hours if the patient has not received antibiotics before collection, and Gram stain of peripheral blood smears often reveals large bacilli in later stages of disease. 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.^{133,141,142}

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%¹⁴³⁻¹⁴⁶ of reported cutaneous anthrax cases and 67% to 94%^{145,146} 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 reported¹⁴⁷ to help with the retrospective diagnosis of anthrax. Western countries have limited experience with this test.¹⁴⁸

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.^{131,149} 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 prolonged treatment, mass prophylaxis, and medication compliance.¹⁵⁰⁻¹⁵⁴ 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.¹⁵⁵⁻¹⁶⁰ In addition, if a bioter-

rorism event occurs, the bacterial strains used may be intentionally antibiotic resistant or genetically modified to confer resistance to one or more antibiotics.

Tetracycline, erythromycin, and chloramphenicol have also been used successfully¹⁶¹ 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.¹⁶²⁻¹⁶⁵ These drugs should be effective in vivo, but there is no reported clinical experience. 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, aminoglycosides, chloramphenicol, imipenem, clarithromycin, and linezolid.^{131,149} 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.^{149,159}

PROPHYLAXIS

Prophylactic Treatment After Exposure

Experimental evidence¹⁶⁶ 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.¹⁶⁷⁻¹⁶⁹

Active Immunization

BioPort Corporation (Lansing, Michigan) 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,^{64,170,171} although this antibody response has not been reported in the limited observations in human vaccinees.¹⁷² Although PA is an effective immunogen,¹⁷³ it is unknown whether the small amounts of LF or EF in some lots of the vaccine contribute to its protective efficacy. The potency of vaccine lots is determined by showing protection of parenterally challenged guinea pigs. An in-vitro assay for vaccine potency is being developed.¹⁷⁴ There is no characterization of the amount and form of the PA or other toxin components in the vaccine. The vaccine

is stored at 2°C to 8°C. The recommended schedule for vaccination is 0.5 mL given subcutaneously over the deltoid muscle at 0, 2, and 4 weeks, followed by boosters of 0.5 mL at 6, 12, and 18 months. Annual boosters are recommended if the potential for exposure continues.

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.^{175,176} More than 500,000 US military personnel have received the licensed anthrax vaccine adsorbed (AVA) vaccine, and no unusual rates of serious adverse events have been noted.¹⁷⁷ Additional studies also support the safety of the anthrax vaccine.¹⁷⁸⁻¹⁸⁶ 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.¹⁴⁷ New attenuated vaccines developed in the United States are being evaluated for efficacy in inhalational anthrax animal models.¹⁸⁷

In the United States vaccination with the licensed vaccine induced an immune response, measured by indirect hemagglutination, to PA in 83% of vaccinees 2 weeks after the first three doses,¹⁸⁸ and in 91% of those tested after receiving two or more doses.¹⁴⁴ 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 (ELISA), the current serologic test of choice, more than 95% of vaccinees seroconvert after the initial three doses.^{172,189}

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.¹⁷⁰⁻¹⁷²

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.^{67,190} A placebo-controlled clinical trial was conducted with a vaccine similar to the currently licensed US vaccine.¹⁹¹ 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.¹⁹² 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.¹⁹²⁻¹⁹⁸ 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^{171,172,195} and an aerosol challenge.¹⁷⁰ The licensed vaccine has also been shown to protect rhesus monkeys against an aerosol challenge.^{166,195,196,198}

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%⁶⁶ and 0.2% to 1.3%.¹⁹² The vaccine licensed in the United States is reported to have a similar incidence of reactions.^{189,199} 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 after they peak. 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 has indicated that frequency of local reactions could be significantly reduced by administering the vaccine over the deltoid muscle instead of the triceps.¹⁷⁷ People who have recovered from a cutaneous infection with anthrax may have severe local reactions from being vaccinated.¹⁹¹ Systemic reactions are characterized by flu-like symptoms, mild myalgia, arthralgia, headache, and mild-to-moderate malaise that last for 1 to 2 days.

There are no long-term sequelae of local or systemic reactions and no suggestion of a high frequency or unusual pattern of serious adverse events.^{177,182,183,200}

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 5

PLAGUE

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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. Plague is foremost a disease of rodents; over 200 species have been reported to be infected with *Y. pestis*.^{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 most 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* caused three great pandemics of human disease. The bubonic form of *Y. pestis* 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; the confluent mass of nodes, if untreated, may suppurate and drain pus); and bacteremia. Septicemic plague can ensue from untreated bubonic plague or without obvious lymphadenopathy after a fleabite. Patients with the bubonic form of *Y. pestis* 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.^{1,6-8} Cervical lymphadenopathy, which is more common in patients from developing countries, may result from flea bites on the neck or face while sleeping on the dirt floors of heavily flea-infested buildings.⁹

During the past four 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 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 plague is not the differential diagnosis, or treatment is absent or delayed.

The US military's concern with plague is both as an endemic disease and as a biological warfare threat. To best prepare 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 currently available for plague, although candidates are in clinical trials. A better understanding of the preventive medicine aspects of the disease will aid in the prompt diagnosis and effective treatment necessary to survive a plague attack.

Key terms in this chapter include enzootic and epizootic. These terms refer, respectively, to plague that is normally present in an animal community but occurs in only a small number of animals, and to widespread plague infections leading to death among susceptible nest populations (ie, equivalent to an epidemic in a human population). The death of a rodent causes the living fleas to leave that host and seek other mammals, including humans. Knowledge of these two concepts helps to clarify how and when humans may be infected, in either endemic or biological warfare scenarios.

HISTORY

The Justinian Plague (First Pandemic)

Procopius provided 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 is now considered the first great pandemic of the CE.¹⁰ At the height of the epidemic, more than 10,000 people died each day. As many as 100 million Europeans, including

40% of Constantinople's population, died during this epidemic.^{11,12} Repeated, smaller epidemics followed this plague.¹³

The Black Death (Second Pandemic)

The second plague pandemic, known as the Black Death, brought the disease into the collective memory of Western civilization.¹³ 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.¹³⁻¹⁵ 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 entered Great Britain at Weymouth.¹⁰

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.¹¹ However, some people believe that the plague persisted through 1720, with a final foray into Marseilles. 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

Failing to understand the plague's epidemiology, 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.¹¹ Physicians recommended a simple diet; avoidance of excessive sleep, exercise, and emotion; regular enemas; and abstinence from sexual intercourse.¹⁶ Although some people killed cats and dogs because they were thought to carry disease, rats seemed to escape attention.¹¹ Christians blamed plague on Muslims, Muslims blamed it on Christians, and both Christians and Muslims blamed it on Jews or witches.¹³

In 1666 a church rector in Eyam, Derbyshire, England, persuaded the whole community to quarantine itself when plague erupted there, but this was the worst possible solution because the people then remained close to the infected rats. The city experienced virtually a 100% attack rate with 72% mortality. The average mortality for the Black Death was consistently 70% to 80%.^{13,17}

Accurate clinical descriptions of the Black Death were written by contemporary observers such as Giovanni Boccaccio in *Decameron*:

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.¹⁸

Marchionne di Coppo di 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 of 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....¹⁹

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."¹¹

The second great pandemic slowly subsided in Europe by 1720. The pandemic's decline was 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 epidemiology mysteries.^{3,8,20}

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.^{13,17} In 1894 Alexandre JE Yersin discovered *Y. pestis* and satisfied Robert Koch's postulates for bubonic plague.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.²¹ The modern pandemic arrived in Bombay in 1898, and during the next 50 years, more than 13 million Indians died of rat-associated plague.^{21,22}

The disease officially arrived in the United States in March 1900, when the lifeless body of a plague-infected Chinese laborer was discovered in a hotel basement in San Francisco, California. The disease subsequently appeared in New York City and Washington state the same year.^{23,24} The plague appeared in New Orleans,

Louisiana, in 1924 and 1926.²⁴ The Texas Gulf Coast and Pensacola, Florida, also saw the influx of plague. Before 1925, human plague in the United States was a result of urban rat epizootics. 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 1925.²⁵ 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 fewer than casualties caused by disease and nonbattle injuries.²⁶ *Y pestis* can initiate disease both through endemic exposure and as a biological warfare agent. Medical officers need to distinguish likely from unlikely cases of endemic disease and consider the possible biological warfare threat.

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.²¹

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 forces were almost free of plague. However, the disease remains on and near military bases in the western United States because the local mammal populations are reservoirs of infection.

World War II

Endemic plague became established in Hawaii (on the islands of Hawaii and Maui) in December 1899. No evidence of the disease, however, in either rodents or humans has been found on the islands of 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 was contained by strict rat control measures that prevented any plague spread to military personnel during the war in the Pacific.²⁷ Official policy during World War II was to

vaccinate US troops with the whole-cell killed plague vaccine. No troops contracted plague, although they served in known endemic areas.^{27,28} Plague has since disappeared from Hawaii.

Vietnam War

Plague entered Vietnam in Nha Trang in 1898 and several pneumonic epidemics have occurred since then.^{21,29,30} Cases have been reported in Vietnam every year since 1898, except during the Japanese occupation in World War II.²¹ When French forces departed Vietnam after the Indochina War, public health conditions deteriorated, and plague flourished. The reported plague incidence increased from 8 cases in 1961 to 110 cases in 1963, and to an average of 4,500 cases annually from 1965 through 1969.^{25,31-34} The mortality in clinically diagnosed cases was between 1% and 5%. In untreated individuals, it was higher (60%–90%).^{21,32} However, only eight American troops were affected (one case per 1 million human-years) during the Vietnam War.³⁴ 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 insect repellent use, protective clothing, and rat-proofed dwellings.^{21,32} During this period, two officers of the US Army Medical Service Corps, Lieutenant Colonel Dan C Cavanaugh and Lieutenant Colonel John D Marshall, 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.^{21,35}

Disease Threat on US Military Installations

Human exposure to plague on military installations may occur at home when pets bring in 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.²⁵ Plague risk has been identified on and near several US military installations (Exhibit 5-1). For a description of relevant rodent/flea complexes found in the United States see the **Epidemiology** section of this chapter.

Plague as a Biological Warfare Agent

The first 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.^{11,21} 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.¹¹ The 20th-century use of plague as a potential biological warfare weapon is of concern and should be considered, 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 by plague because it could create casualties out of

EXHIBIT 5-1

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, Colo: 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.

proportion to the number of bacteria disseminated, the most dangerous strains could be used to make a very dangerous 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.³⁶

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 too low for safety. However, clay bombs solved these technical difficulties and resulted in an 80% survival rate of fleas.³⁶

At 5:00 AM on a November day 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 was observed falling from the plane. Within 2 weeks, individuals in that area of the city 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 mortality was not noted until months **after** the human cases. It was also observed that plague usually spreads with rice (because rats infest the grain) along shipping routes, but the nearest epidemic center was 2,000 km away by land or river. Changteh exported, not imported, rice. These unusual circumstances surrounding the

plague outbreak suggest that it may have been of human origin.³⁶

In another incident, on October 4, 1940, a Japanese plane dropped rice and wheat grains mixed with fleas over the city of Chuhsien in the 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 the 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 increased mortality was found in the rat population.³⁶

Since World War II

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.³⁷ 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."³⁷ 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 groups may likely consider obtaining plague for use as a biological weapon.

During the Korean War, allied forces were accused of dropping insects that could spread plague, typhus, malaria, Japanese B encephalitis, and other diseases on North Korea. However, no evidence supports such claims.³⁸

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*.³⁹ 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*.⁴⁰ This proposal was not well received, primarily because of 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 millennia, during which the enzootic "pestoides" isolates evolved (see **Biochemistry** below). The pestoides strains appear to have split from *Y pseudotuberculosis* over 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.⁴¹

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 5-1). Depending on growth conditions, *Y pestis* can exhibit marked pleomorphism with rods, ovoid cells, and short chains present. A gelatinous envelope, known as the F1 capsular antigen, is produced by the vast majority of strains at a growth temperature of 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.^{39,42}

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 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% CO₂. The round, moist, translucent, or opaque colonies are nonhemolytic on sheep blood agar and exhibit an irregular edge. 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.^{20,39,42} Strains to be archived should be grown at low temperatures and frozen promptly at –70°C.

Biochemistry

Y pestis is a facultative anaerobe, fermenting glucose with the production of acid. An obligate pathogen, 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 and ferment glycerol.²⁰ Some molecular methods of typing, such as ribotyping and restriction fragment-length polymorphisms of insertion sequence locations, support this division of strains.^{43,44} 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⁺) is found in Central Asia and Africa and may represent the most ancient

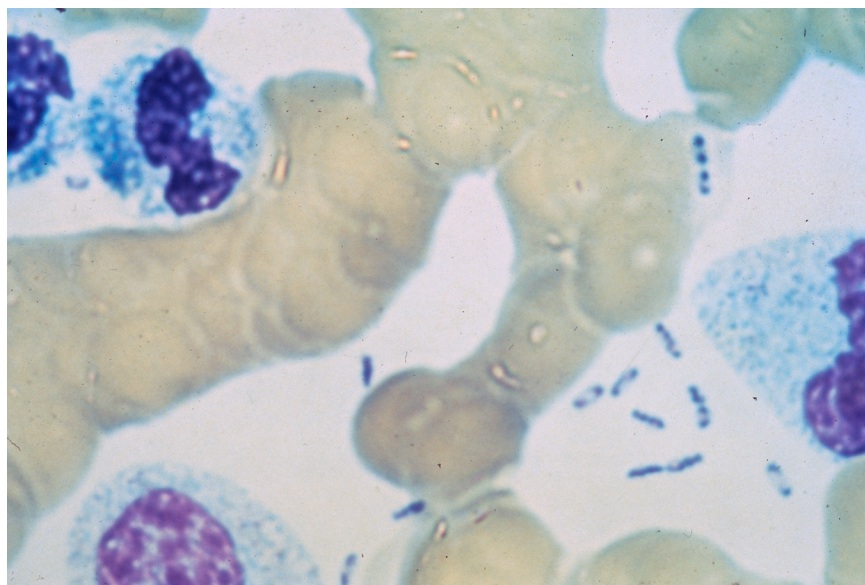


Fig. 5-1. This Wright-Giemsa stain of a peripheral blood smear from a patient with septicemic plague demonstrates the bipolar, safety-pin staining of *Yersinia pestis*. Gram’s and Wayson’s stains can also demonstrate this pattern. Photograph: Courtesy of Kenneth L Gage, PhD, Centers for Disease Control and Prevention Laboratory, Fort Collins, Colorado.

of the biovars.^{20,41} Biovar *mediaevalis* (Gly⁺, Nit⁻) is geographically limited to the region surrounding the Caspian Sea. No apparent differences in pathogenicity exist among the biovars.^{20,45} Recently, three different multilocus molecular methods were used to investigate the microevolution of *Y. pestis*. 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*.⁴⁶ These isolates represent the most ancient of the *Y. pestis* strains characterized to date.⁴¹

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.²¹ 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 relative few species play a significant role in disease transmission.^{25,47,48} *Y. pestis* is not capable of blocking (see below) all flea species and there appears to be variability in the ability of various flea species to transmit the organism.⁴⁸

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.¹⁰ After the flea ingests a blood meal from a bacteremic animal, bacilli multiply and eventually block the flea's foregut, or proventriculus, with a fibrinoid mass of bacteria as shown in Figure 5-2.²¹ When feeding, the flea ingests approximately 0.03 mL to 0.5 mL of blood. 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 blockage. Blockage limits feeding resulting in repeated 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.²¹ 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.^{21,26}

Although the largest plague outbreaks have been associated with *X. cheopis*, all fleas should be considered dangerous in plague-endemic areas.^{2,48} 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 have been found to contain *Y. pestis*.¹⁰ However, the presence of plague bacilli in these latter insects is associated with ingestion of contaminated blood from

plague victims, and they apparently had little or no role as vectors for the disease. The most important vector of human plague in the United States appears to be *Oropsylla montana*, the most common flea on rock squirrels and California ground squirrels,²⁵ 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.

The black rat, *Rattus rattus*, has been most responsible worldwide for persistence and spread of plague in urban epidemics throughout history. *R. rattus* is a nocturnal, climbing animal that does not burrow, but instead nests overhead and lives in close proximity to humans.¹⁰ In the United Kingdom and much of Europe, the brown rat, *R. norvegicus*, a burrowing

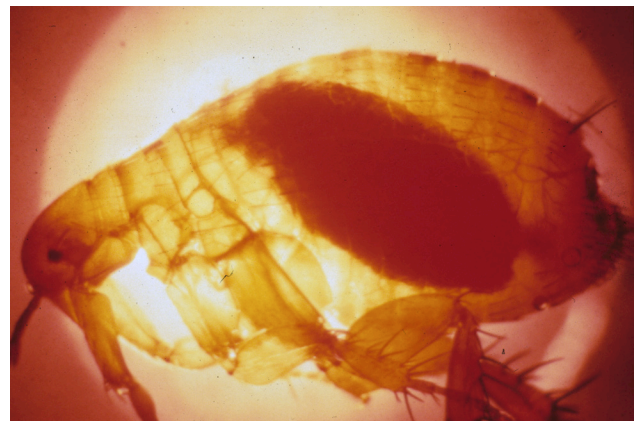


Fig. 5-2. The oriental rat flea (*Xenopsylla cheopis*) has historically been most responsible for the spread of plague to humans. This flea has a blocked proventriculus, equivalent to a human's gastroesophageal region. In nature, this flea would develop a ravenous hunger because of its inability to digest 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.

animal that lives under farm buildings and in ditches, has replaced *R rattus* as the dominant city rat.⁴⁹ 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.¹⁰ Most carnivores, except cats, are resistant to plague infection, but animals such as domestic dogs, all rodents, and burrowing owls may transport infected fleas into homes. Mammals that are partially resistant to plague infection are continuous plague reservoirs. Some epidemiologists propose that the true plague hosts are rodent species with populations consisting of both sensitive and resistant individuals, but others

have questioned the need for resistant individuals within the species to maintain plague foci.⁵⁰ In the United States, prairie dogs (*Cynomys* species) and *Spermophilus* species (rock squirrels and ground squirrels) are most often associated with plague activity. A variety of susceptible mammals, such as chipmunks, tree squirrels, cottontail rabbits, and domestic cats (Figure 5-3), are occasionally infected. Epizootic spread among tree squirrels in Denver recently resulted in the first urban plague case since the 1920s.⁴⁷ Although not associated with any human plague cases, the appearance of two infected fox squirrels in Dallas, Texas, in 1993 also caused considerable concern.⁵¹ An increasing

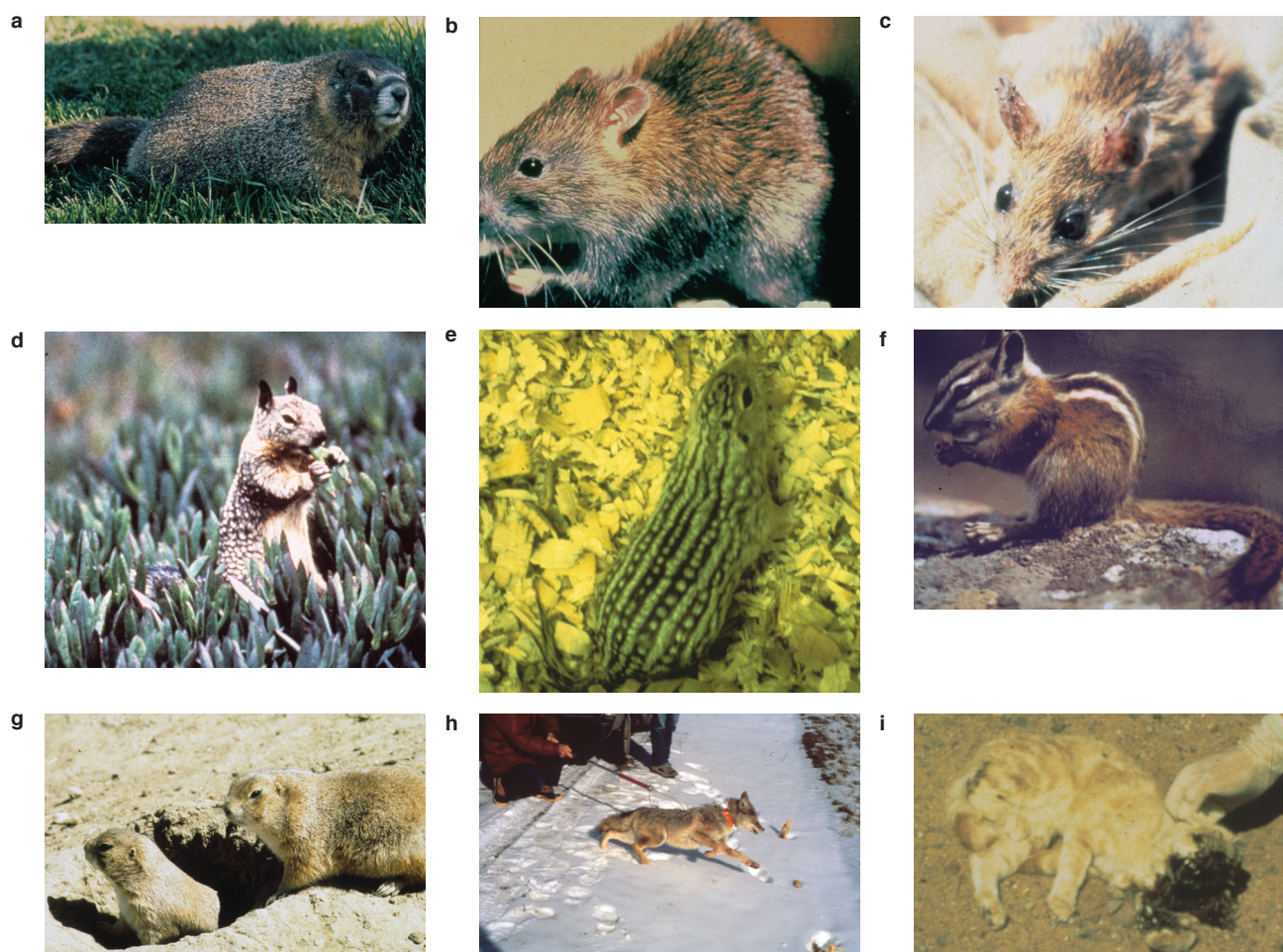


Fig. 5-3. Known mammalian reservoirs of plague in the United States (noninclusive). The common North American marmot (a) and the brown rat (*Rattus norvegicus*) (b), which has largely replaced the black rat, are considered to be reservoirs of plague (ie, hosts to infected fleas). Other reservoirs of plague during enzootics are thought to include the deer mouse (c), the California ground squirrel (d), and the 13-lined ground squirrel (e). Other infective mammals that can spread plague to humans include the chipmunk (f), prairie dogs (g), and the coyote (h). Domestic and nondomestic cats are also reservoirs of plague. This cat (i), which died of pneumonic plague, demonstrates a necrotic head. Photographs a, h: Courtesy of Denver Zoological Society, Denver, Colorado. Photographs b-g, i: Courtesy of Centers for Disease Control and Prevention Laboratory, Fort Collins, Colorado.

number of human infections has been associated with domestic cats, usually through bites, contact with tissues, suppurating buboes, or aerosol rather than by transmission of fleas.⁴⁷ Cats appear to be particularly efficient at transmitting disease to humans.^{47,48}

Highly susceptible animals amplify both fleas and bacilli and often support the spread of epizootics, especially when these animals occur at high densities.⁵² In many developing countries, these epizootics often involve commensal rat species (*Rattus*). 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 served as sources of infection for humans, who acquired the disease by handling infected prairie dogs. Rock squirrels and California ground squirrels both infect humans via direct contact and fleas.^{10,25,53,54} Many other mammals in the United States harbor plague, and a few, including wild carnivores such as bobcats, have served as sources of infection for humans (Exhibit 5-2). Knowledge of local host species is important, because certain mammal–flea complexes found in the United States are particularly dangerous: these complexes consist of both a susceptible mammal and a flea known to bite humans. More than one host-epizootic complex can occur in a given area. These pairings include the following^{25,48}:

EXHIBIT 5-2

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, Colo: US Army Center for Health Promotion and Preventive Medicine, Fitzsimons Army Medical Center; September 1995: 25–28. Technical Guide 103.

- the rock squirrel (*S variegatus*) or California ground squirrel (*S beechyi*) and the flea *Oropsylla montana*, which is known to readily bite humans;
- the antelope ground squirrel (*Amмосpermophilus leucurus*) and the flea *Thrassus bacchi*;
- the prairie dog (*Cynomys* species) and the flea *Opisocrostis hirsutus*;

PLAGUE CYCLES IN THE UNITED STATES

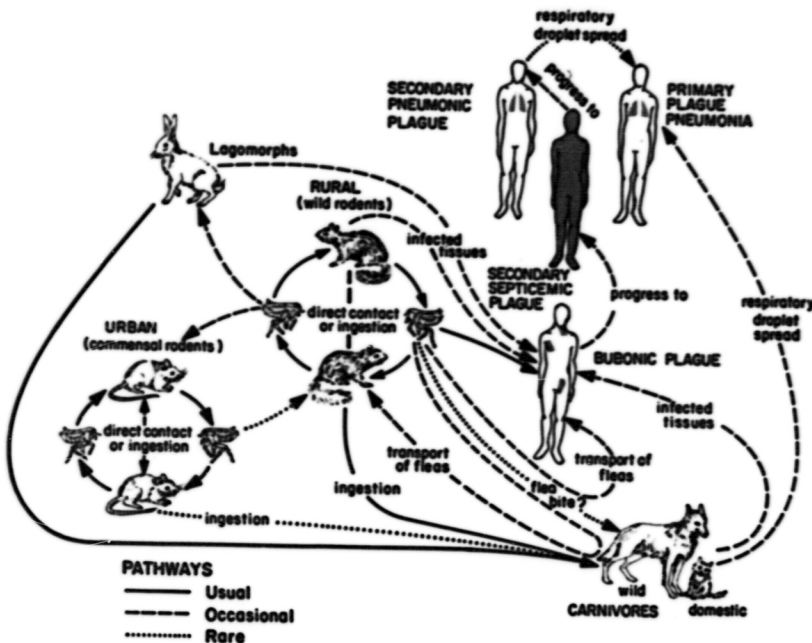


Fig. 5-4. Plague cycles in the United States. This drawing shows the usual, occasional, and rare routes by which plague is known to have spread between various mammals and humans.

Reproduced with permission from Poland JD. Plague. In: Hoeprieh PD, Jordan MC, eds. *Infectious Diseases: A Modern Treatise of Infectious Processes*. Philadelphia, Pa: Lippincott; 1989; 1297.

- the Wyoming ground squirrel (*Spermophilus richardsoni*) or the golden-mantled ground squirrel (*S. lateralis*) and the fleas *Opisocrostis labis*, *Opisocrostis idahoensis*, or *Thrassus bacchi*; and
- various wood rat species (*Neotoma sp*) and the fleas *Orchopeas sexdentatus* and *Orchopeas neotomae*.

Plague exists in one of two states in nature: (1) enzootic or (2) epizootic. An enzootic cycle is a stable rodent–flea infection cycle presumably occurring in a relatively resistant host population and not causing excessive rodent mortality. When the disease is in an enzootic cycle, the fleas have no need to seek less desirable hosts—such as humans. During an epizootic, however, plague bacilli have been introduced into moderately or highly susceptible mammals. High mortality occurs, most conspicuously in larger colonial rodents such as prairie dogs.¹ These epizootics are most likely to occur 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.⁵⁵ Humans are accidental hosts in the plague cycle and are not necessary for the persistence of the organism in nature (Figure 5-4).

INCIDENCE

World Health Organization (WHO) member states are required to notify WHO of human plague cases under the International Health Regulations, although the policy on bubonic plague in endemic areas may soon change. Plague may be significantly underreported for several reasons, including the reluctance of some endemic countries to admit public health problems, difficulties in diagnosis, and the absence of laboratory confirmation. From 1989 to 2003, 38,310 cases (with 2,845 deaths) were recorded in 25 countries, with the highest number of human plague cases reported in 1991 and the lowest number in 1989. Generally, the distribution of plague coincides with the geographical distribution of its natural foci.^{57,58}

Plague is endemic in many countries in Africa, the former Soviet Union, the Americas, and Asia. In 2002 human plague was reported in Africa (Democratic Republic of the Congo, Madagascar, Malawi, Mozambique, Uganda, and the United Republic of Tanzania), the Americas (Peru and the United States), and Asia (China, India, Kazakhstan, Mongolia, and Vietnam). The vast majority of these cases (approximately 95%) were in Africa. Since the early 1990s, increasing reports of plague in Africa may represent an increase in disease or an improvement in notification to WHO. Recent

Humans typically acquire plague via infectious bites of fleas. Infection via flea feces inoculated into skin with bites may also occur. Less common sources of infection include human fleas, contact with tissues from an infected animal, consumption of infected tissues, handling of contaminated pelts, and respiratory droplet transmission from animals with pneumonic disease.^{1,3,21,47,48} Humans bitten by fleas during the grooming behavior practiced in some cultures have also been implicated in some plague cases. 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.²¹ Human-to-human plague transmission can occur from patients with pulmonary infection. However, the understanding of pneumonic plague epidemiology is incomplete. Most 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 epidemics. The role of particle size in efficiency of transmission is unknown, although it may occur more efficiently via larger droplets or fomites rather than via small-particle aerosols.⁵⁶

resurgence of plague in India, Indonesia, and Algeria during the past decade has occurred after “silent” periods of 30 to 50 years.^{57,59} Worldwide distribution of plague and its epidemiology can be found in the WHO’s Plague Manual available online at <http://www.who.int/csr/resources/publications/plague>. Recent reports of plague activity and occasional summaries of plague activity can be found at the Web sites for WHO’s Weekly Epidemiological Record (<http://www.who.int/wer/en/>) and the Centers for Disease Control and Prevention’s Morbidity and Mortality Weekly Report (<http://www.cdc.gov/mmwr/>). Known foci of plague are shown in Figure 5-5.

Recently, 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 for two cases of the septicemic form, were reported as pneumonic plague. No evidence of bubonic disease was observed. Multidisciplinary health teams from WHO investigated the potential outbreak, but no report has been issued since March 2005.⁶⁰ The prevalence of pneumonic disease in the group of cases (assuming that this was plague) has not yet been explained.

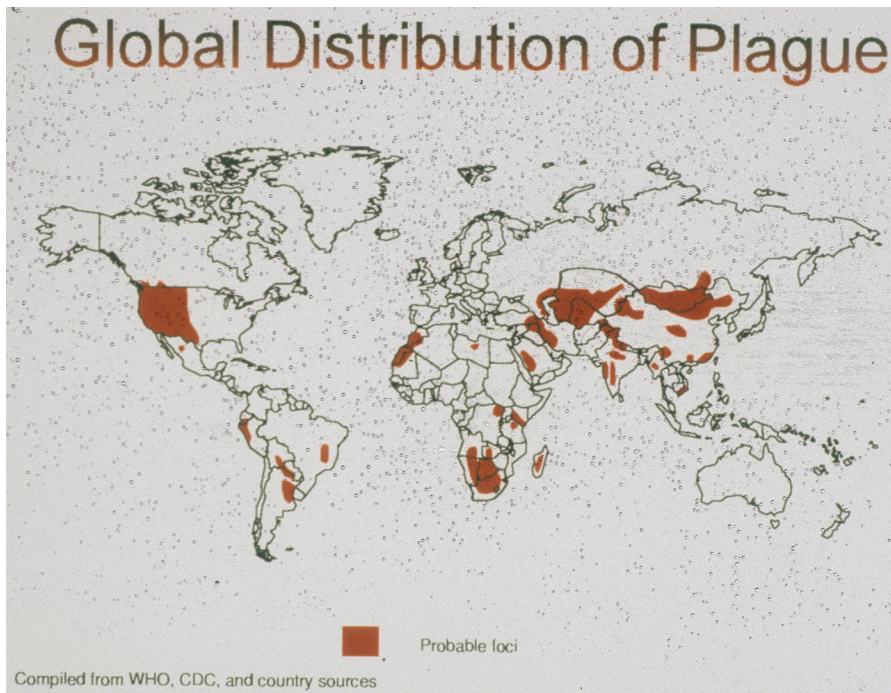


Fig. 5-5. Known worldwide foci of human plague infection. Data sources: (1) Human plague in 1990. *WHO Weekly Epidemiological Record*. 1 Nov 1991;44:321–324. (2) Human plague in 1993. *WHO Weekly Epidemiological Record*. 17 Feb 1995;7:45–48. (3) Barkway J. World Health Organization, Geneva, Switzerland. Personal communication, February 1996. (4) Kenneth L Gage, PhD, Centers for Disease Control and Prevention Laboratory, Fort Collins, Colorado. Personal communication, March 1996.

Plague has been endemic in the continental United States since at least 1900 and now is permanently established from 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.^{2,24,25,48} In the first quarter of this century, virtually all 432 cases and 284 deaths (65.7% mortality) in the United States occurred in urban port cities. Epidemics occurred in San Francisco, California, during 1900 through 1904 (118 deaths) and 1907 through 1908 (78 deaths). The last time plague was transmitted between humans in the United States was during the 1924 through 1925 pneumonic plague epidemic in Los Angeles, California. Eighty percent of cases since 1925 have been sylvatic, involving contact with wild rodent habitats.²⁴ Most cases (58%) are in men and occur within a 1-mile radius of home, and half of the victims in the United

States have been younger than 20 years old.^{24,25}

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; it then decreased to 9 per year since 1990.⁵³ 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.²⁵ Most human plague cases are reported from New Mexico, Arizona, Colorado, and California.^{51,61} Epizootic cycles occur approximately every 5 years; the last extremely widespread epizootic with a large die-off of rodents over multiple states (in 1982–1984) was accompanied by the highest number of humans infected with plague since the urban epidemics of the first quarter of the 20th century.^{53,54}

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 different hosts. To maintain the transmission cycle, *Y pestis* must 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 that of the midgut of the flea, 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 body temperature of this host; presumably additional factors also cue the organism to recognize this environment and respond

appropriately. Likewise, genes required for replication in the mammalian host are expressed at highest levels at 37°C; and the synthesis of some proteins, thought to be induced in the phagolysosome, is also regulated by pH. In the laboratory, growth temperature and calcium concentration control both the synthesis and secretion of certain essential virulence factors; the induction of these proteins has been termed the low calcium response (LCR).^{2,20,62,63}

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: (1) pMT (or pFra), which encodes the F1 capsule, and (2) 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 a number of antihost factors, is an absolute requirement for virulence.²⁰

Type III Secretion System

Like a number of other gram-negative pathogens, the human pathogenic *Yersiniae* 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.^{64,65} In the case of the *Yersinia* species, this system is encoded on the pYV plasmid, which encodes the components of the LCR. Toxic activities of the LCR effector proteins, designated Yops (*Yersinia* outer protein), 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.⁶⁶ 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 antiinflammatory role by inhibiting inflammatory cytokine production and inducing apoptosis in macrophages.^{63-65,67} Overall, the effect is that of paralyzing professional phagocytes, and it is clear why the pathogen–host interaction mediated by the type III secretion system has been designated the “*Yersinia* Deadly Kiss.”⁶⁸

LcrV (historically known as V [or virulence] an-

tigen), 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: (a) as regulator of Yop transcription, (b) for translocation of Yops into the host cell, and (c) as a virulence factor in its own right.^{20,63} LcrV appears to stimulate production of the immunosuppressive cytokine interleukin 10 through interactions with Toll-like receptor 2 and CD14 signaling. These effects appear to be mediated by the N-terminal portion of LcrV.^{69,70}

The secretion mechanism includes an “injectisome” that can be visualized as a needle-like structure by electron microscopy. Another group of proteins promotes the secretion process by forming pores in the mammalian cell membrane. 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 a secretion channel that allows the Yops to be translocated through the membrane and into the host cell.^{64,65}

F1 Capsular Antigen

The F1 capsule, 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. This gelatinous envelope is generally thought to protect the organism from host phagocytic cells by interfering at the level of receptor interaction in the phagocytosis process,⁷¹ and 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 F1 capsular antigen, F1-negative strains have been isolated from rodent hosts and reportedly from one human case.⁷²⁻⁷⁵ In the laboratory, spontaneous mutants defective in F1 production have been obtained from immune animals, from cultures treated with antiserum containing F1 antibody, and from chronically infected rodents.⁷²⁻⁷⁴ 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.^{72,76} 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, the studies indicate 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 F1, 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*. It is the major antigen recognized in convalescent sera of humans and rodents,^{77,78} and is also a highly effective protective immunogen.

Other Virulence Factors in the Mammalian Host

The virulence factor plasminogen activator (Pla) is encoded by 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.^{20,79} Pla is necessary for full virulence in some *Y pestis* strains. However, a few strains that are Pla-negative and appear to be fully virulent have been identified among natural isolates or generated in the laboratory.^{20,80,81} Presumably, these isolates synthesize other proteins that substitute for Pla function.

The so-called pH 6 antigen is a fimbral 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, which 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.⁸² More recent data, however, suggest that the pH 6 antigen does not enhance adhesion to mouse macrophages but rather promotes resistance to phagocytosis.⁸³

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 many bacteria, *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 yersiniabactin, a low-molecular-weight iron chelator, or those unable to transport yersiniabactin are not capable of growth in mammals.^{20,84} The genes encoding this iron transport system are situated on a chromosomal pathogenicity island with the Hms locus (see below).

Virulence Factors in the Flea

Researchers have begun to address the factors that allow *Y pestis* to block the flea and promote vectorborne transmission. Both chromosomal and plasmid-encoded gene products have been found to play roles in flea blockage. One of these loci, Hms, is expressed only at temperatures lower than 28°C; bacteria producing the Hms-encoded outer membrane protein are hydrophobic and form aggregates in vitro. Although Hms mutants are capable of colonizing the flea midgut, they are unable to colonize the proventriculus and, therefore, do not block the flea. Hms-mediated aggregation promotes formation of a biofilm that allows the organism to persist in the proventriculus despite the shearing forces that flush nonaggregating cells into the midgut.⁸⁵ Hms 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 does not appear to play a role in mammalian plague. 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 is responsible for attenuation.²⁰

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.⁸⁶ 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.⁸⁶ This may be explained by the regulation of toxin synthesis. Like Hms, murine toxin is produced more efficiently at 28°C than at mammalian body temperatures.

PATHOGENESIS

As few as 1 to 10 *Y pestis* organisms are sufficient to cause infection by the oral, intradermal, subcutaneous, or intravenous routes.⁸⁴ Estimates of infectivity by the respiratory route for nonhuman primates vary from

100 to 20,000 organisms.^{76,87,88}

After being introduced into the mammalian host by a flea, the organism is thought to be initially susceptible to phagocytosis and killing by neutro-

phils. However, some of the bacteria may grow and proliferate within tissue macrophages.⁸⁹ The relative importance of intracellular versus extracellular replication in plague has been extensively debated. 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. They suggest that, although considerable attention has been 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.⁹⁰ Once the antiphagocytic gene products are expressed, the bacteria are resistant to phagocytosis and multiply extracellularly.

During the incubation phase, the bacilli most commonly spread to regional lymph nodes, where suppu-

rative lymphadenitis develops, producing the characteristic bubo. Dissemination from this local site leads to septicemia and seeding of other organs, including 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 other causes of gram-negative sepsis. The endotoxin may also contribute to the resistance of the organism to the bactericidal activity of serum.⁹¹

Primary pneumonic plague, the most severe form of disease, arises from inhalation of an infectious aerosol. Primary pneumonic plague is more rapidly fatal than secondary.¹

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 been reported to occur in contacts of patients with either bubonic or pneumonic plague.^{92,93}

CLINICAL MANIFESTATIONS

From 1947 through 1996, 390 cases of plague were reported in the United States, resulting in 60 deaths (15.4%). 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%). Seven cases (1.8%) were unclassified, including one (death 14.3%).^{51,61} If *Y pestis* was used as a biological warfare agent, the clinical manifestations of plague would be (a) epidemic pneumonia with rapid progression and a high fatality rate if aerosolized bacteria were used or (b) bubonic or septicemic plague—or both, 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).²¹ Six to 8 hours after onset of symptoms, buboes, heralded by severe pain, occur in the groin (femoral and inguinal lymph nodes), axillary, or cervical lymph nodes—depending on the site of bacterial inoculation (Figure 5-6). Buboes, which are 1 cm to 10 cm in diameter, have overlying erythematous skin and are so painful that nearly comatose patients

attempt to shield them from trauma and abduct their extremities to decrease pressure. Buboes are often associated with considerable surrounding edema, but lymphangitis is rare. Occasionally, buboes can become fluctuant and suppurate. A small minority of patients bitten by plague-infected fleas develop *Y pestis* septicemia without a discernible bubo. Other manifestations of bubonic plague include bladder distention, apathy, confusion, fright, anxiety, oliguria, and anuria. Tachycardia, hypotension, leukocytosis, and fever are frequently encountered. Untreated bubonic plague can result in septicemia 2 to 6 days later, which, if left untreated, is virtually 100% fatal.⁹⁴ In the United States, approximately 10% to 15% of bubonic plague patients will develop secondary pneumonic plague with the potential for airborne transmission of the organism.⁹⁵

Septicemic Plague

Septicemic plague may occur primarily, or secondarily, as a complication of hematogenous dissemination of bubonic plague. 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 5-7), disseminated intravascular coagulation, and acral cyanosis and necrosis, particularly of the extremities (Figure 5-8), 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 plague,



Fig. 5-6. 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.



Fig. 5-7. 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.



Fig. 5-8. 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.

which indicates the difficulty of diagnosing septicemic plague.⁹⁶ Diagnosis of septicemic plague took longer (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 attributable to hepatosplenomegaly.⁹⁶

Pneumonic Plague

Pneumonic plague may occur primarily, from inhaling aerosols, or secondarily, from hematogenous dissemination. Patients with pneumonic plague typically have symptoms of a severe bronchopneumonia, dyspnea, cough, chest pain, and hemoptysis.^{97,98} The

findings on chest roentgenography may be variable, but bilateral alveolar infiltrates appear to be the most common finding (Figure 5-9).^{99,100} The sputum, which may be clear, purulent, or hemorrhagic, contains gram-negative rods. Unless appropriate antimicrobial therapy is begun on the first day of illness, pneumonic plague is rapidly fatal. The time from respiratory exposure to death in humans is reported to have been between 2 and 6 days in epidemics during the preantibiotic era.⁹⁷

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.¹⁰¹

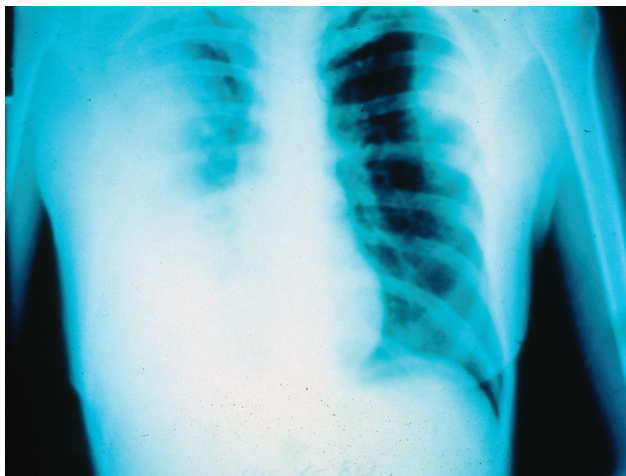


Fig. 5-9. This chest roentgenogram shows right middle- and lower-lobe involvement in a patient with pneumonic plague.

Photograph: Courtesy of Kenneth L Gage, PhD, Centers for Disease Control and Prevention Laboratory, Fort Collins, Colorado.

Pharyngeal Plague

Asymptomatic transient pharyngeal carriage has been reported to occur in healthy contacts of bubonic plague cases.^{92,93} Rarely, pharyngitis resembling tonsillitis and associated with cervical lymphadenopathy has been reported.^{6,94} A plague syndrome of cervical buboes, peritonsillar abscesses, and fulminant pneumonia has also been reported to occur among Indians of Ecuador, who catch and kill fleas and lice with their teeth. Endobronchial aspiration from peritonsillar abscesses is suspected to lead to fulminant pneumonia. A similar syndrome may have also occurred in Vietnam.⁹⁴ Consuming meat from infected camels and goats is also implicated in the development of disease.^{1,3}

Cutaneous Manifestations

Approximately 4% to 10% of plague patients have an ulcer or pustule at the inoculation site (Figure 5-10).¹⁰⁰⁻¹⁰² The flea typically bites the lower extremities; therefore, femoral and inguinal buboes are the most common. Infection arising from skinning plague-infected animals typically produces axillary buboes. Buboes may point and drain spontaneously or, rarely, they may require incision and drainage because of pronounced necrosis. Petechiae and ecchymoses may occur during hematogenous spread to such an extent that the signs mimic severe meningococcemia, with



Fig. 5-10. 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.

similar lesions. The pathogenesis of these lesions is probably that of a generalized Shwartzman reaction (disseminated intravascular coagulation secondary to the *Y pestis* endotoxin). When purpura and acral gangrene occur, possibly resulting from the activities of the plasminogen activator, the prognosis is poor.^{21,103} Patients in the terminal stages of pneumonic and septicemic plague often develop large ecchymoses on the back. Lesions like these are likely to have led to the medieval epithet "the Black Death." Ecthyma gangrenosum has been reported in several patients.^{88,103} A sample from a case of ecthyma gangrenosum grew *Y pestis*, which suggests that the skin lesions resulted from septicemic seeding of the organism.¹⁰³

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 healthcare provider is unfamiliar with plague or if the patient presents in a nonendemic area or without a bubo, the diagnosis may be delayed or missed. When

a bubo is present, the differential diagnosis should include tularemia, cat scratch disease, lymphogranuloma venereum, chancroid, tuberculosis, streptococcal adenitis, and scrub typhus (Figure 5-11). In both tularemia and cat scratch disease, the inoculation site is typically more evident and the patient is usually not 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 have a recent history of sexual contact and

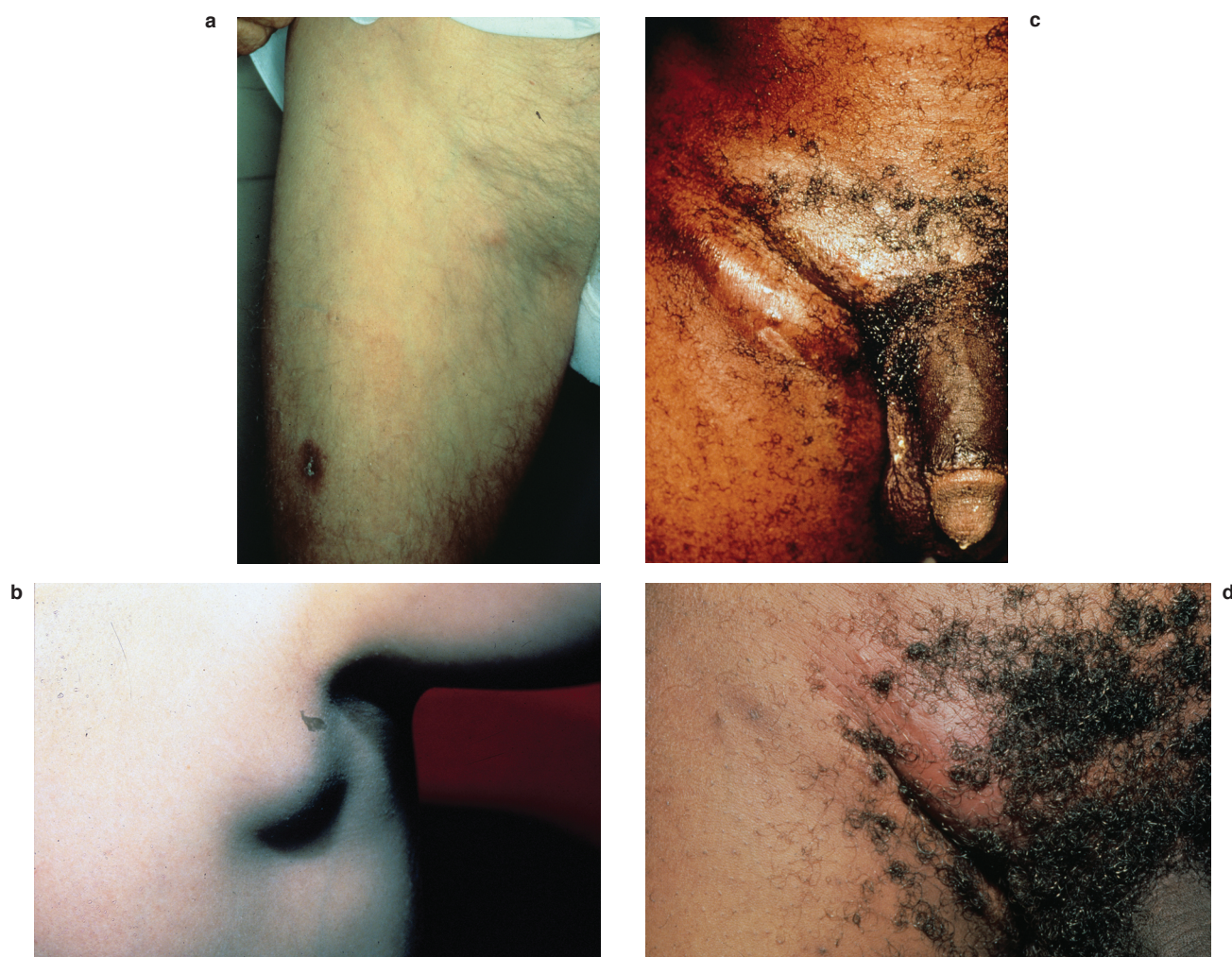


Fig. 5-11. (a) Small femoral bubo and presumed inoculation site (on the inferior thigh) in a patient with tularemia. This gram-negative bacterial infection (with *Francisella tularensis*) may closely mimic bubonic plague and is successfully treated with the same antibiotics. (b) Axillary bubo seen in 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.

genital lesions. Those with the latter disease may be as sick as patients with plague. Streptococcal adenitis may be difficult to distinguish from plague initially, but the patient is not usually septic, and the node is more tender when plague is present.

Implications of the absence of a bubo were discussed in a review of 27 plague cases seen in New Mexico.¹⁰⁰ In this study, there were 8 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 development was associated with a delay in the diagnosis of plague and increased mortality.¹⁰⁰

The differential diagnosis of septicemic plague also includes meningococemia, 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 lead to 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 Centers for Disease Control and Prevention Web site (<http://www.bt.cdc.gov/agent/plague/index.asp>).¹⁰³ The World Health Organization offers its *Plague Manual* online at <http://www.who.int/emc-documents/plague/whocdscsredc992c.html>.⁵⁹ A recent review of the methodology for isolating and identifying *Y pestis* from clinical samples and animals is available.⁴² Standard bacterial methodologies include staining and microscopic analysis of the organism, isolation on culture medium, and biochemical tests. Laboratories experienced in the identification of *Y pestis* with the appropriate containment facilities should perform diagnostic tests for plague. Care should be taken to avoid aerosols; in this regard, fixing slides with methanol rather than heat fixing is preferred.

Reference laboratories, such as those found in major county or state health departments, have additional tests to confirm the diagnosis of *Y pestis*. These include direct fluorescent antibody tests to detect the F1 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 Serologic tests 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 Centers for Disease Control and Prevention, Fort Collins, Colorado; and the US Army Medical Research Institute of Infectious Diseases at Fort Detrick, Maryland.¹⁰⁴ Serologic assays measuring the immune response to plague infection are mainly of value retrospectively because patients present clinically before they develop a significant antibody response.

When using the fluorescent antibody test to detect the plague-specific F1 capsular antigen, it is important to realize that F1 is produced only at temperatures greater than 33°C. Thus, this method requires a relatively fresh sample from the patient or 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-negative.⁴² 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 test detected concentrations of F1 antigen as low as 0.5 mg/mL in as little as 15 minutes and had a shelf life of 21 days at 60°C. It had 100% sensitivity and specificity against laboratory isolates of *Y pestis*, and the agreement between field-testing and reference laboratory testing was 89.9%. The test demonstrated positive and negative predictive values of 90.6% and 86.7%, respectively.¹⁰⁵ 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 the rapid diagnosis of plague 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 the surveillance of rats and could be adapted to help diagnose human infection.¹⁰⁶ The use of *Pla* primers for simulated detection of *Y pestis* in sputum was reported recently to have a sensitivity of 10⁴ colony forming unit/mL and a 5-hour turnaround.¹⁰⁷ When trying to determine whether *Y pestis* has been used as a biological weapon, it should be kept in mind that F1 or *Pla* are not necessary for virulence in animal models,^{72,76,80} and strains lacking these important diagnostic targets could be seen.

Cultures of blood, bubo aspirate, sputum, and cerebrospinal fluid (if indicated) should be performed. Tiny, 1- to 3-mm "beaten-copper" colonies 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.⁹⁷ 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 remembered 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 5-12).⁵⁸

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. When disseminated intravascular coagulation is present, fibrin degradation products are elevated. Because of liver involvement, alanine aminotransferase, aspartate aminotransferase, and bilirubin levels are often increased.

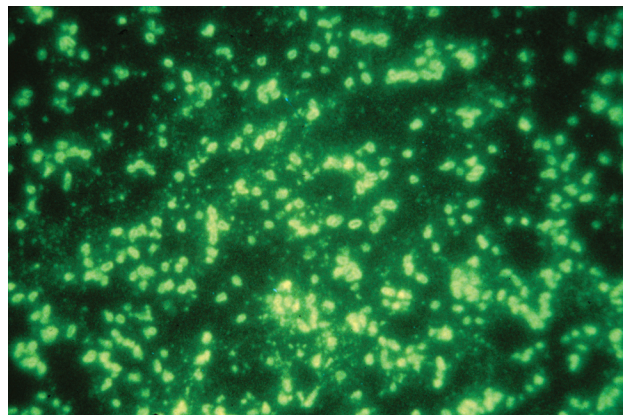


Fig. 5-12. These *Yersinia pestis* fluorescent cells are from an infected mouse spleen. Notice how the outlines of the coccobacilli “light up” in this direct fluorescent antibody (DFA) test. The DFA test is specific and therefore better than the other stains discussed in this chapter (original magnification $\times 1,000$).

Photograph: Courtesy of MC Chu, Centers for Disease Control and Prevention Laboratory, Fort Collins, Colorado.

TREATMENT

Isolation

All patients with plague should be isolated for the first 48 hours after the initiation of treatment. Special care must be taken in handling blood and bubo discharge. If pneumonic plague is present, then strict, rigidly enforced respiratory isolation procedures must be followed, including the use of gowns, gloves, and eye protection. Patients with pneumonia must be isolated until they have completed at least 48 hours of antibiotic therapy and have a favorable clinical response. If patients have no pneumonia or draining lesions at 48 hours, they may be taken out of strict isolation. Microbiology laboratory personnel must be alerted when *Y pestis* is suspected (four laboratory-acquired plague cases have been reported in the United States).¹⁰⁸

Antibiotics

Since 1948 streptomycin has remained the treatment of choice for bubonic, septicemic, and pneumonic plague. Streptomycin is approved by the Food and Drug Administration for treatment of plague. Streptomycin should be given intramuscularly in a dose of 30 mg/kg per day in two divided doses for 10 days. However, streptomycin is rarely used in the United States, and supplies of this antibiotic are limited.¹⁰⁹ The Working Group on Civilian Biodefense recommends

gentamicin as an alternative to streptomycin even though it is not approved by the Food and Drug Administration for treating plague. 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.¹¹⁰ Although there are no controlled comparative trials, in a recent review of 75 human plague cases in New Mexico researchers concluded that gentamicin alone or in combination with a tetracycline was as efficacious as streptomycin for treating humans infected with plague.¹¹¹ Alternative 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).¹¹⁰ Chloramphenicol is indicated for conditions in which high tissue penetration is important, such as plague meningitis, pleuritis, or myocarditis; it can be used separately or combined with an aminoglycoside. In pregnant women, the preferred choice is gentamicin with doxycycline or ciprofloxacin as alternatives. Streptomycin should be avoided in pregnant women if possible.¹¹⁰ The treatment of choice for plague in children is streptomycin or gentamicin. Doxycycline, ciprofloxacin, or chloramphenicol are recommended as alternatives by the Working Group on Civilian Biodefense.¹¹⁰ Chloramphenicol should not be used on

children less than 2 years old due to the risk of “grey baby syndrome.” 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.¹¹⁰ In patients 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 the onset of symptoms. Without treatment, mortality is 60% for bubonic plague and 100% for the pneumonic and septicemic forms.⁹²

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.^{21,31} Fleas must always be targeted before rodents, because killing rodents may release massive amounts of infected fleas.⁹⁵ 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 through constant preening.²¹ Under International Health Regulations, plague must be reported to WHO as an internationally quarantinable disease for which travelers may be detained for up to 6 days. However, because of ongoing revisions in the International Health Regulations, bubonic cases in endemic areas may no longer be subject to mandatory reporting.⁹

Postexposure Prophylaxis

Asymptomatic individuals such as family members, healthcare providers, or others who have had close contact with persons with untreated pneumonic plague should receive antibiotic prophylaxis for 7 days. Close contact is defined as contact with a patient at less than 2 m.¹¹⁰ Prophylaxis is also recommended for laboratory workers exposed to an accident, which may have created an infectious aerosol. Doxycycline is the preferred antibiotic, given as 100 mg twice daily for 7 days. Ciprofloxacin or chloramphenicol are alternatives. The Working Group for Civilian Biodefense recommends that people who develop fever or cough while receiving postexposure prophylaxis seek prompt medical attention and begin parenteral antibiotic treatment.¹¹⁰ Hospital personnel who are observing recommended isolation procedures do not require

prophylactic therapy, nor do people in contact with patients with bubonic plague. However, people who were in the same environment and who were potentially exposed to the same source of infection as the patients with plague should be given prophylactic antibiotics. The Centers for Disease Control and Prevention 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 known to be 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 was responsible for the multidrug-resistant phenotype of this isolate, suggesting a potential for transfer to other *Y pestis* strains in nature.¹¹² Russian scientists have published descriptions of multidrug-resistant plague vaccine strains produced in the laboratory; these techniques also could conceivably be used on virulent strains.¹¹³ Ciprofloxacin-resistant isolates have been obtained in the laboratory from attenuated strains.¹¹⁴ If *Y pestis* is used as a biological weapon, antibiotic resistance is a possibility.

Vaccination

In 1897 Russian physician Waldemar MW Haffkine developed the first plague vaccine consisting of killed whole cells in India. 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, that 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.^{87,88,115-117}

Two new plague vaccine candidates that use the F1 and V antigens of *Y pestis* have been developed. F1 appears to prevent phagocytosis of plague bacilli,

and 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).¹¹⁸ 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.¹¹⁹ The

separate proteins are then combined, two parts F1 to one part V, to form a subunit vaccine. F1-V vaccine has been shown to protect African green monkeys from pneumonic plague.¹¹⁸ Both vaccines are in clinical trials, although the Porton vaccine is somewhat more advanced in the process. After further testing, it is conceivable that one of these vaccine candidates may be selected for further development as a human vaccine candidate that could protect against pneumonic plague.

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. By contrast, in the most likely biological warfare scenario, plague would be spread via aerosol. Person-to-person spread of fulminant pneumonia, characterized by blood-tinged sputum, would then ensue. If, however, an enemy force were to release fleas infected with *Y pestis*, then soldiers would pres-

ent with classic bubonic plague before a die-off in the local mammalian reservoir occurred.

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 aerosols from an infected human or animal are inhaled.

Diagnosis is established by isolating the organism from blood, sputum, or other fluids or tissues. Rapid diagnosis may be made with fluorescent antibody stains of sputum or tissue specimens. Patients should be isolated and treated with aminoglycosides. Chloramphenicol should be added when meningitis is suspected or shock is present. A licensed, killed, whole-cell vaccine is no longer available. New vaccines that appear to protect against pneumonic plague are being considered for development.

Acknowledgments

We thank the reviewers for their thoughtful comments and Ms Katheryn Kenyon for editorial assistance. 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 civil servants.

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Chapter 6

GLANDERS

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INTRODUCTION

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SUMMARY

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INTRODUCTION

Glanders, a highly contagious and often fatal zoonotic disease of solipeds, including horses, mules, and donkeys, is caused by infection with the bacterium *Burkholderia mallei*. Glanders is characterized by ulcerating granulomatous lesions of the skin and mucous membranes. Disease progression and pathology in humans and horses are similar, although the clinical presentation of any two cases in the same species—even if related by direct transmission—may vary significantly.¹⁻⁴ Generalized symptoms include fever, myalgia, headache, fatigue, diarrhea, and weight loss. After infection, the organism generally travels through lymph channels, first to regional lymph nodes often causing irritation (lymphangitis, lymphadenitis) en route. Unchecked, organisms may enter the bloodstream and travel throughout the body. Without proper treatment, the disease course may range from acute and rapidly fatal to slow and protracted with alternating remissions and exacerbations.

Glanders, an old disease that was described toward the beginning of recorded history, is less commonly

known by other names, including equinia, malleus, droes, and farcy. Farcy is an ancient term for a particular cutaneous manifestation of glanders that before 1882 was believed to be a separate disease of horses. With this cutaneous manifestation of glanders, nodular abscesses (farcy buds) become ulcerated, and regional cutaneous lymphatic pathways become thickened and indurated (farcy pipes) and ooze a glanders-typical yellow-green gelatinous pus (farcy oil).⁵ Pure farcy without ulceration of the mucous membranes was rare—if not just a temporary stage of glanders infection—as was vice versa.³ Humans, goats, dogs, cats, rabbits, and carnivorous predators living close to infected equids or carcasses have been naturally infected.^{2,6} Camels have also been infected and are associated with human disease.⁶ Naturally occurring glanders has been eradicated in most countries, but is still found in parts of Africa, the Middle East, South America, and Eastern Europe. *B mallei* 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 launched 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 shipped from the United States to the Allies were inoculated with cultures of *B mallei*.⁷ In 1914 Anton Dilger, a member of the German army and an American-educated surgeon, was sent home to live with his parents in Virginia after a nervous breakdown. He brought strains of anthrax and glanders and, with his brother's help, set up a laboratory 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.

Also, 4,500 mules in Mesopotamia were infected with glanders by German agents; a German agent was arrested in Russia with similar intentions in 1916; and French cavalry horses were also targets for intentional glanders infection.⁸ Germany and its allies infected many mules and horses on Russia's eastern front, which successfully impaired artillery movement and troop and supply convoys. Concurrent with this increase in animal cases during and after the war, human cases increased in Russia. Attempts to contaminate animal feeds in the United States were also made. A report by

the Monterey Institute of International Studies states that between 1932 and 1945 Japan developed *B mallei* as a biowarfare agent, infecting 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 1937.⁹ The former Soviet Union was alleged to have used weaponized *B mallei* against opposition forces in Afghanistan between 1982 and 1984.¹⁰

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. Glanders 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 is the largest reported human case series in US medical literature.¹ The seventh case has not been previously published. 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

In 1972 the United States signed the Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin

Weapons and on Their Destruction, which banned development, production, stockpiling, acquisition, and retention of biological agents, toxins, and the weapons to deliver them.⁸ All offensive biological warfare work at Fort Detrick had ceased by that time; any remaining biological weapons were destroyed by 1973. Research aimed at the biodefense of *B mallei* warfare is currently being conducted in the United States. There are no known current attempts for acquisition and use by terrorists.¹²

B mallei was considered a potential threat agent in 1947 because of its high infectivity, high degree of incapacitation among those infected, and agent availability.¹³ It poses a more significant threat if weaponized. As exemplified by past clusters of laboratory-acquired infections, *B mallei* is infectious by the respiratory route, but it is not contagious among humans. A determined bioterrorist could likely gain access to the agent,

whether from an infected animal, laboratory culture, or commercial culture. Because glanders is relatively unknown in the West and its clinical symptoms are protean and nonspecific, diagnosis and treatment may be delayed postattack, even in regions with the most advanced medical facilities. Delayed diagnosis and treatment could lead to significant morbidity and mortality. Treatment may be complicated by the relative scarcity of knowledge and experience in therapy. Because equids and some other animals are susceptible, further spread from animals to humans could occur long after an attack. Glanders is curable, and postexposure prophylaxis may be an option after an attack. As with other agents, genetic engineering could produce unpredictable virulence and atypical antibiotic resistance. If glanders were cultivated, concentrated, and delivered as a wet or dry bacterial aerosol, significant casualties could result.¹⁴

HISTORY

Aristotle first described glanders in horses in 330 BCE, and named it malleus, meaning hammer or mallet. Glanders was associated with various horse populations around the globe, particularly army horses and mules. The association of glanders with domesticated equids was so familiar that "horses and their glanders" commonly appeared together in early literature. Glanders was not studied systematically until the 19th century. In 1882 the causative agent now called *B mallei* was isolated from a glanderous horse's liver and spleen.² The first account of the disease in humans was published in 1821,³ yet the medical community recognized it earlier as a syndrome. The first veterinary school was established in Lyon, France, in the mid-1700s to study rinderpest and glanders. Many researchers at the school became infected and died of glanders.¹⁵

Horses and mules were the primary modes of transportation in all developing economies until the Industrial Revolution. Particularly in urban locations, glanders passed from the infected to the uninfected animals housed in crowded conditions. Horses and mules were in high demand during the American Civil War. Thousands of animals passed through remount stations where glanders existed in epidemic proportions. The problem was exacerbated after the war, when glanders was spread to communities as infected military stock was sold to civilians. 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 meticulously disinfecting 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 cases increased countrywide. The notion of a carrier-state began to be accepted. Despite epidemic disease in equine populations, no simultaneous epidemics occurred in the human population.

Vaccines and therapeutic agents were developed but were unsuccessful in reducing the glanders incidence. By 1890 the mallein diagnostic skin test was developed. Control and eradication programs soon incorporated the testing of all contact equids, followed by quarantine and a recommendation for slaughter of all skin-test-positive animals. These programs failed in some locales at first because of lax enforcement and lack of incentive to owners for killing their nonclinically ill animals. Some horse owners hid contact animals to avoid testing, or they sold contact and asymptomatic test-positive animals to unsuspecting individuals to minimize their economic loss.⁴ Inexpensive steam transportation aided disease spread when glanders carriers were shipped to other regions and countries. The United States was blamed for the import of glanders-infected horses to Cuba in 1872³ and for the great increase of glanders cases in Canada, where tens of thousands of US horses were shipped annually, near the turn of the 20th century.^{3,4}

Once control programs offered indemnity to test-positive and contact animals and people 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

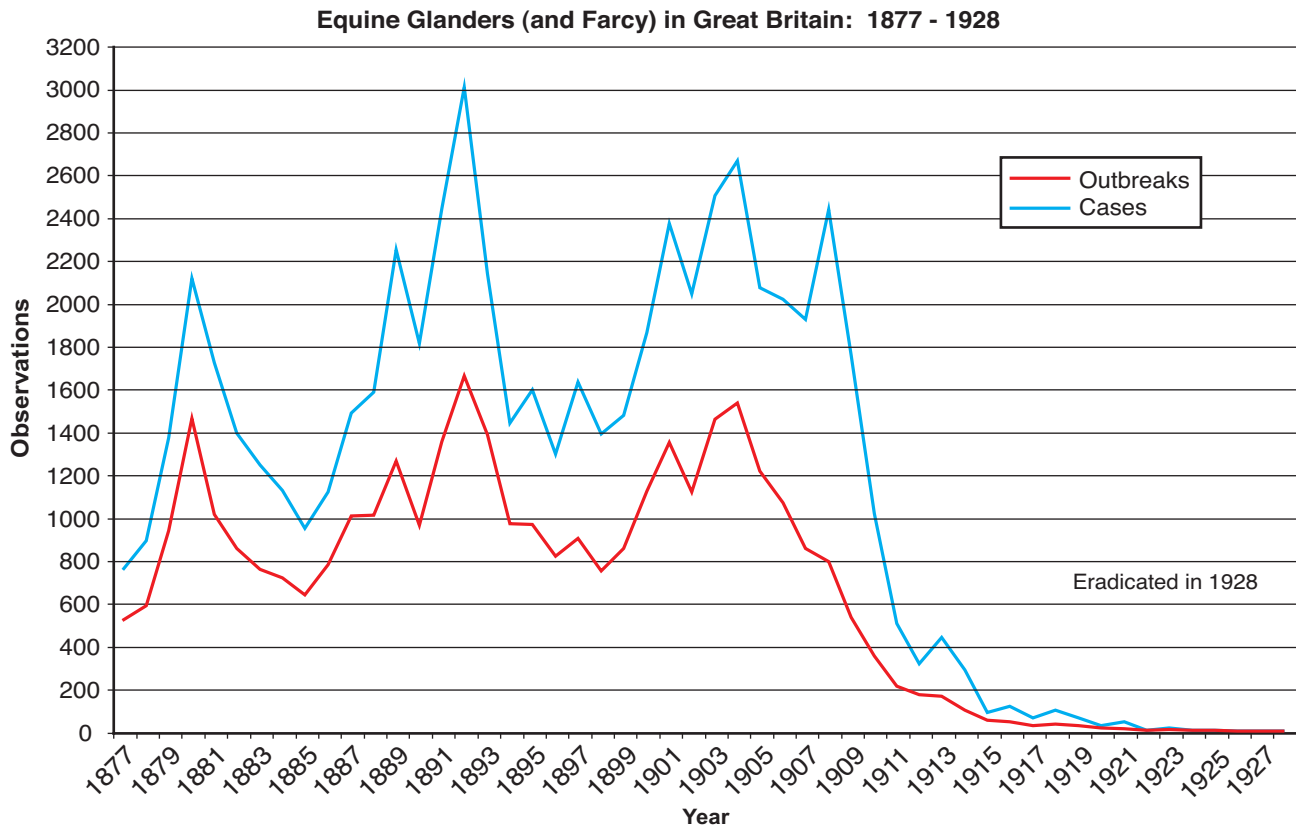


Fig. 6-1. Glanders cases and outbreaks reported to the Department for Environment, Food, and Rural Affairs in Great Britain, 1877–1928. Glanders was eradicated in Great Britain in 1928. Data source: Available at: <http://www.defra.gov.uk/animalh/diseases/notifiable/glanders/index.htm>.

experience with the rise and fall of glanders outbreaks in equids¹⁶ typifies many countries as shown in Figure 6-1. Great Britain eradicated glanders by 1928, about 30 years after eradication programs were initiated. The United States eradicated glanders by 1942.¹⁷ The last naturally occurring human case was recorded in 1934.

Glanders is a zoonotic disease of concern internationally and is notifiable to the 164-member Office International des Epizooties (OIE) in accordance with the International Animal Health Code.¹⁸ Several countries still have eradication programs. In over 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. Between 1996 and 2003, glanders in livestock was reported in Bolivia, Belarus, Brazil, Eritrea, Ethiopia, Iran, Latvia, Mongolia, Myanmar, Pakistan, and Turkey. During the same time frame, glanders in humans was reported in Cameroon, Curaçao, Sri Lanka, Turkey, and the United States (laboratory-acquired).¹⁷ Exhibit 6-1 depicts the year equine glanders was last reported to the OIE among countries and territories without glanders activity (by OIE report) since 1996. Bioterrorism should be considered as a possible source if confirmed glanders is found in the countries and territories listed in Exhibit 6-1.

INFECTIOUS AGENT

Glanders is caused by *B mallei*, a gram-negative bacillus that is a close relative to *B pseudomallei* (causative agent for melioidosis). *B mallei* is an obligate animal pathogen¹⁹ and has not been found free-living in the environment; however, *B pseudomallei* can be isolated from tropical soil. The lack of motility is

a primary means of differentiating *B mallei* from *B pseudomallei*. Growth requirements are not complex; *B mallei* can be cultivated on basic nutrient medium, and glycerol can be added to the medium to enhance growth. When stained, the cells typically exhibit bipolar staining.

EXHIBIT 6-1

YEAR EQUINE GLANDERS WAS 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	Norway	1889
Croatia	1959	Poland	1957
Denmark	1928	Portugal	1952
Egypt	1928	Romania	1960
Estonia	1945	Serbia and Montenegro	1959
Finland	1943	Slovakia	1954
Yug Rep of Macedonia (former)	1957	South Africa	1945
France	1965	Spain	1956
Georgia	1960	Sudan	1989
Germany	1955	Sweden	1943
Greece	1965	Switzerland	1937
Hungary	1956	Taipei China	1950
India	1988	Great Britain	1928
Ireland	1920	Northern Ireland	1910
Israel	1951	United States of America	1942
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 2003. (Data are available only for the listed countries and territories.)
OIE: Office International des Epizooties

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*,²⁰ and was finally assigned to the genus *Burkholderia* in 1992.²¹ Not particularly hardy in the environment,²⁰ *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 for 1 month.^{2,16,22} Experimentally and under the most favorable temperature and moisture conditions, Loeffler extended the viability of *B mallei* to 100 days. In nature, the organism's viability 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. Because of its high infectivity by aerosol, laboratory studies on this Category B pathogen²³ are performed at biosafety level 3 (BSL-3). Varying degrees of virulence among strains have been shown in the laboratory and in nature.^{1,4,6} The infectious dose is low, depending on the route of infection, susceptibility, and strain virulence. One to 10 organisms of some strains by aerosol are lethal to hamsters.^{1,24} Inhaling only a very few organisms may cause disease in humans, equids, and other susceptible species.

DISEASE

Epidemiology

Naturally acquired cases of glanders in humans or equids are sporadic and rare; most countries have eradicated the disease. Glanders is still infrequently reported in northern Africa, the Middle East, South America, and Eastern Europe.¹⁷ Serologic cross-re-

activity with *B pseudomallei* precludes the accurate distribution and prevalence of *B mallei* by serologic means alone. Although human outbreaks have been reported in Austria and Turkey, no human epidemic has been recorded.²⁵

In nature, the horse is the reservoir of *B mallei* and may also be the amplifying host. A disease primarily of

solipeds, donkeys are considered most prone to develop acute forms of glanders, and horses are more prone to develop chronic and latent disease. Mules, a crossbred animal resulting from a horse and donkey, are susceptible to both acute and chronic disease as well as latent infections.^{20,26,27} 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.²⁴ 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. Veterinarians and veterinary students, farriers, flayers (hide workers), transport workers, soldiers, slaughterhouse personnel, farmers, horse fanciers and caretakers, and stable hands have been naturally infected. Subclinical or inapparent infections in horses and mules pose a hidden risk to humans. Human-to-human transmission is rare. Infection by ingesting contaminated food and water has occurred; however, it does not appear to be a significant route of entry for human infections.^{2,6,28} 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.¹

Transmission

Glanders is transmitted directly by bacterial invasion of the nasal, oral, and conjunctival mucous membranes by inhalation into the lungs and by invasion of abraded or lacerated skin. The arms, hands, and face are most often exposed. Considering the affinity for warm and moist conditions,² *B mallei* may survive longest in stable bedding, manure, feed and water troughs (particularly if heated), wastewater, and enclosed equine transporters. Transmission from handling contaminated fomites, such as grooming tools, hoof-trimming equipment, harnesses, tack, feeding and husbandry equipment, bedding, and veterinary equipment, has occurred. Such equipment stored away from any contact with equids for at least 3 months—even without disinfection—is not likely to be a source of infection.

Reports of the circumstances surrounding zoonotic transmission are diverse. A few reports include equids snorting in the vicinity of humans or human food, and humans wiping equine nasal exudate off their arm with a blade of grass (local infection occurred at wipe site), sleeping in the same barn or stall as apparently healthy equids, accidentally puncturing themselves with contaminated equipment, wiping an eye or nostril after contact with an equid, being licked by a glandered horse, and cleaning stalls without any direct equine contact.^{3,29,30} Horse handling requires physical work that often produces skin abrasions under normal circumstances. Although absorption through intact skin is probably unlikely, patients may insist their skin was intact when exposed. Among 105 people with chronic glanders associated with equid exposure described by Robins,³ only 40 (38%) reported a wound present. In 27 cases (17%) the absence of a wound was specifically noted.

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 when mouth pipetting was a common practice. The first six patients acknowledged using this technique to clear blocked pipettes and blow contents out of pipettes that were calibrated to the tip. The eighth case involved a microbiologist who had worked with *B mallei* in BSL-3 containment for 2 years, but did not always wear latex gloves.¹¹ 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. Most laboratory-acquired infections are not associated with injury or a recollection of injury.³¹ This patient had diabetes for 13 years, however, and collected blood via finger-stick morning and evening. 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 has occurred. The majority of reported events occurred in medical practice, at autopsy, in the diagnostic laboratory, and in patient care settings before a clearer understanding of universal precautions existed.^{2,3,11} Transmission also occurred in home settings, where close contact during care of glanders-infected individuals led to infection of other family members.³ At least one entire family became infected: the two children and wife of a chronically infected stable hand contracted glanders. The wife was presumably infected sexually;

the 4-year-old 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.

Human infection by ingestion has not been definitively reported. Stomach contents can inactivate *B mallei* experimentally in 30 minutes.²⁵ In his detailed 1886 report on the etiology of glanders, Loeffler describes several accounts of humans eating meat from glanderous horses without contracting disease. In one account, over 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 setting at that time. In another case, a veterinarian ate raw glanderous meat to answer the ingestion question, but did not contract disease. An 1886 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.² Monogastric animals, including lions, tigers, domestic cats, dogs, and bears, have died from *B mallei* infection from ingesting raw meat.² 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 through the healthy digestive tract. This explanation, however, does not explain infections in dogs, domestic cats, and captive wildlife that were fed only boneless meat from glanderous horses. From this limited collection of testimonies and understanding of glanders pathogenesis, it appears that human ingestion of the live organism is unlikely to cause disease.

These features of transmission exemplify the requirement for BSL-3 containment and safety practices when working with *B mallei*. Laboratory workers should adhere to safety procedures and universal barrier precautions. In the presence of potentially infected equids, transmission risk is also reduced by universal precautions and procedures that reduce inhalation risk of potentially contaminated aerosols. The advances in medicine, infection control, and therapeutics make it less likely now than 100 years ago for human-to-human transmission to occur, even in a human outbreak, whether related to bioterrorism or not. It is also highly unlikely that an equid reservoir will become established. Acute disease is expected to manifest in a significant proportion of exposed equids, which would necessitate emergency response, quarantine, trace-back, 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.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. Sharing feed and water troughs facilitates this transmission,^{20,26,27} as well as common equid behaviors that include grooming and snorting. Because equids are unable to breathe through their mouths, simple exhalation—and in particular, snorting to clear nasal passages—can finely aerosolize infectious nasal efflux from an infected equid. This snorting poses an absolute 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.² Sexual transmission from stallion or jack to mare or jenny has also occurred. The breeding of asymptomatic stallions resulted in the spread of glanders near the turn of the 20th century.⁴

Carnivores can become infected after eating contaminated carcasses and meat.³² Reported outbreaks in captive wild felids suggest that they appear to be more susceptible than canids.^{20,26,32,33} Glanders has also been transmitted to goats housed with infected horses.² Laboratory animals including mice, hamsters, guinea pigs, rabbits, and monkeys are also susceptible.^{2,34} Cattle, swine, and chickens appear to be resistant to glanders, even after experimental injection.^{2,33} Pigeons have been infected experimentally.² Loeffler suggested that field mice, donkeys, mules, horses, goats, cats, and guinea pigs were more susceptible to glanders infection and clinical disease than humans. Among other susceptible host species, rabbits and dogs appeared to be less susceptible to disease than humans.

Pathogenesis

The clinical course and potential chronicity of glanders show *B mallei* to be a hardy and persistent organism in situ that can evade attack from the immune system. The cytoplasmic membrane and cell wall consist of three layers.²⁴ In experimentally injected guinea pigs, *B mallei* produces a tenacious capsule that may protect it from phagocytosis.³⁵ The structure of the capsule described in this study is unknown. However, more recent genetic analysis has shown that the coding sequence of the *B mallei* capsule is 99% identical to the carbohydrate capsule encoded by *B pseudomallei*, which is a homopolymer of

-3)-2-O-acetyl-6-deoxy-β-D-manno-heptopyranose-(1-^{36,37} Furthermore, a mutant strain without the capsule is avirulent in mice and hamsters.³⁶ The capsule does not stain with typical capsule stains. The organism has an affinity for the lymphatics and can be found within and outside the host cell. Where there are glandular nodes of infection even deep within the musculature, ulceration and drainage to the outside of the body generally occur; internal organs are an exception. Some strains of *B mallei* produce an endotoxin that affects smooth muscle cells of various organs.⁶ Tissue reactions, including lymphangitis and mucous membrane erosions, and the slow healing nature of local infections are clinical symptoms that support this local effect.

Acute and chronic glanders infections were described long before a viable treatment was available and before most countries had eradicated the disease. In his 1906 review of 156 chronic human glanders cases, Robins stated that distinguishing 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 Robins defined chronic cases as those lasting longer than 6 months. Most historical literature attempting to distinguish between the two in humans and equids classifies a more fulminant and rapidly fatal clinical course (within 2–4 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.^{1,25,38} Chronic infections are most common in horses, where they comprise the majority of cases.⁶ An acute disease course is more common in donkeys and humans.

B mallei most often enters the human body through abrasions or openings in the skin, particularly on the hands and forearms, face, and neck, where occupational exposure occurs. 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 there was no wound or penetration during the likely exposure interval. 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, which has occurred in several laboratory-acquired infections. However, at least one laboratory-acquired case most likely occurred through cutaneous exposure. When they are present, the most characteristic feature of the disease is glanders nodes, small papular to egg-sized abscesses, which are slow to heal if they open.

The incubation period 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; although without direct inoculation of the organism, the duration may be longer.³ Inhalational exposure may incur a slightly longer range of about 7 to 21 days.^{1,3}

Clinical Disease in Animals

B mallei naturally infects horses, donkeys, and mules,^{20,39} although other species have occasionally become infected.^{32,40} 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 varies from a few days to many months, most often falling between 2 and 6 weeks. The infectious process, disease progression, and pathology in equids are similar to those in humans. Donkeys are most likely to die from acute disease within a week to 10 days.^{2,4} 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 increases temperature, such as infectious disease, roundup, transport, overwork, poor diet, exercise, vaccination, and even mallein testing.^{2,4,41} 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, as well as by direct close contact with infected animals.⁴² 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 very gregarious, preferring 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. Tack such as a harness can cause skin irritation that, if the tack is contaminated, may allow easy entry of the organism. Despite the oral route of infection, significant pathology is usually seen in the airways and lungs.¹⁹

With early infection or recurrence, constitutional signs are often the first to manifest including thirst, fever (low-grade to high), shivering, drooping of the head, tachycardia, tachypnea, weight loss, rough hair coat, indolence, prostration, and reluctance to move.⁴³ Limbs and joints may swell. 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.² In some cases (or at various disease stages), 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 characterized at least 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. However, laryngeal, tracheal, and lower respiratory tract pathology is often present, 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.^{3,30}

With clinical expression of upper respiratory infection, a highly infectious, sticky, yellow-gray to greenish viscous unilateral or bilateral nasal exudate is produced. The glottis may be edematous and the thickness of nasal discharge may 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 from 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, which 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 the aid of endoscopy and are near-hallmark signs of prior infection. Visible or palpable regional lymphadenopathy (particularly submandibular) and lymphangitis are usually present.

The equid frequently snorts 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 because equids breathe through their nose, not their mouths. Dyspnea, particularly

inspiratory, may result from swelling in the nasal cavity or larynx. Expiratory dyspnea is also common, particularly with chronic involvement of the upper and lower respiratory tract.²⁹ 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. Animals may die within a few days, or within 3 to 4 weeks from bronchopneumonia and septicemia.

At necropsy, glanders nodes are likely found in the lungs, even if incidentally. Their consistency may be caseous to calcified depending on lesion age. These nodes may be 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.

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 into more solid, fingerlike cords that can be traced to regional lymph nodes. Nodules along the lymphatic pathways 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. The lesions are 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, which may result in behavioral changes such as avoidance or fear. With disseminated disease, cutaneous and mucous membrane lesions may appear anywhere, particularly in the respiratory tract as previously mentioned, and on the limbs. The hind limb is more commonly affected than the forelimb.^{22,26}

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 donkeys that are naturally and experimentally infected. In horses, however, disseminated disease is typically more protracted. Clinical signs are widely variable and may include any of those previously mentioned. Horses may be asymptomatic, or 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 male donkeys may have orchitis, which may not be evident without a reproductive examination.^{20,44} 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 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 eradication (by test-and-slaughter) of the disease.

Chronically infected horses may display cycles of worsening disease followed by apparent recovery when few symptoms are displayed. Clinical signs include intermittent cough; lethargy; and lesions in the nasal region, lungs, and skin, just as with acute disease.⁴³ Lungs may develop lesions similar to tubercles. Nodules may appear in the submucosa of the nasal cavity, particularly in 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.⁴⁵ Attempts to isolate *B mallei* from chronically infected animals are usually unsuccessful. Thromboses can be found in the large venous vessels of nasal mucous membranes.⁴⁶ Nodules in the skin along lymphatics may be seen as they thicken in chronically infected animals. Nodules may ulcerate and rupture, spewing a thick exudate that can 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 be variable. At least six forms of infection have been described, including

nasal, localized (the nasal form is also a localized form), pulmonary, septicemic, disseminated, and chronic infection; none are exclusive. The most important distinction is whether the infection is truly localized, which is unusual except early in the infectious process. The variety of forms is largely explained by various routes 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. Clinical courses will be discussed in detail below because they are associated with route of entry and disease spread.

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 pathways that drain the entry or infection site. Mucus production from affected ocular, nasal, and respiratory mucosa is often increased. Localized infections typically disseminate, leading to pulmonary, septicemic, or disseminated infection.

Constitutional signs and symptoms typically occur early in the course of disease, and some may persist through treatment and be severe, leaving the patient exhausted. 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 (including night sweats); altered mental status; and fatigue. Other nonspecific signs, any of which may be present, include tender lymph nodes, sore throat, chest pain, blurred vision, splenomegaly, abdominal pain, photophobia, and marked lacrimation.

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, although 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.^{6,29} Localized infection with a mucopurulent discharge develops at the entry site. Inflammation may extend along regional lymphatics and cause lymphangitis with numerous foci of sup-

puration along their course. This irritation is caused by endotoxins present in some *B mallei* strains affecting the smooth muscle of the lymphatics. Lymphatic pathways 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 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 studies have described glanders nodes anywhere, including the face, neck, shoulders, lumbosacral region, arms, and legs. 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. The 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 mucous 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 infected, swell, and weep a serosanguineous 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 (affecting the 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 there may be copious nasal discharge. Infection may invade the nasal septum and bony tissues, causing fistulae and tissue destruction. The face may swell, and regional lymph glands may inflame and suppurate. Infection may also extend lower in the respiratory tract, resulting in tracheitis and bronchitis that may be accompanied by cough and mucopurulent sputum production. If mucous membrane involvement is extensive, consti-

tutional signs, such as fever, severe headache, fatigue, prostration, earache, and various neurologic signs are also usually severe.

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, such as fatigue, fever, chills, headache, myalgias, and gastrointestinal signs, often accompany respiratory infections. Pulmonary abscess and pleuritis are common sequelae. Symptoms, which may take up to 2 to 3 weeks to develop, include tender cervical lymph nodes, fatigue, lymphangitis, sore throat, pleuritic chest pain, cough, fever (often exceeding 102°F), chills, tachypnea, dyspnea, and mucopurulent discharge. Nonspecific signs, such as night sweats, rigors, myalgia, severe headache, tachycardia, nausea, weight loss, dizziness, and mucosal eruptions, are also usually present. 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. Untreated acute bronchopulmonic or pneumonic disease has a rapid onset of symptoms and was once said to be almost uniformly fatal within 10 to 30 days.¹ Most laboratory-acquired infections have been caused by inhalational exposure resulting in pulmonary infection.

Clinical features of eight laboratory-acquired infections from Camp (later Fort) Detrick are summarized in Table 6-1. 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 (see Table 6-1). 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

TABLE 6-1
CLINICAL FEATURES OF EIGHT US LABORATORY-ACQUIRED *B 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		+						
Oro-pharyngeal	Postnasal drip	Blister under tongue; nasal obstruction				Sore throat		
Pharynx injected	+	+			+			
Lymphadenopathy	Cervical		Cervical	-	Cervical			L axilla
Neurologic signs			Stupor	Carpopedal 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, pneumonitis	L-hilum ~Abscess	Clear

(Table 6-1 continues)

Table 6-1 continued

WBC	Normal-low; neutropenia	Normal	High; neutro- philia	High to normal to low; Neutro- phils	Normal	Normal to high- normal; Neutro- phils	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 ^s	Day 50	Day 50	Day 12	Day 40	-	-	-	NA
Agglutinin positive ^y	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	Sulfa- diazine 10 days	Sulfa- diazine 10 days	Sulfa- diazine 36 days	Sulfa- diazine 20 days	Sulfa- diazine 20 days	Sulfa- diazine 20 days	Aureo- mycin 28 days	Doxycy- cline 6.5 mos
Onset of antibiotic [¶]	Day 60	Day 60	Day 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 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.

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.

^s CFTs were considered positive if \geq 1:20.

^y Agglutinin titers were positive if \geq 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

NA: not applicable or not done

WBC: white blood cell

for patient 2. Inhalation is suspected as the route of exposure for the first seven patients, and 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 in chronic or latent infection. Septicemia

may be passing and lead to protracted disseminated infection or be fulminant and rapidly fatal. Without aggressive treatment, *B mallei* septicemia runs an acute course and may lead to death in 7 to 10 days. Septicemic glanders may produce numerous signs consistent with a highly pathogenic bacterial septicemia. The thromboembolic process of glanders was

well described by the early 1900s.^{2,3} *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 patient may recognize the embolic process 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. However, death may occur before these manifestations develop. Clinical signs and symptoms of the septicemic process may develop immediately or up to 2 weeks after initial infection or recurrence. 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. Patients die within 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.⁵ Dissemination typically occurs without adequate treatment 1 to 4 weeks after *B mallei* infection of the lymph nodes. The organs most 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 or limited to weight loss, or they may be severe, variable, and include any of those mentioned earlier. Cutaneous eruptions may appear 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 pathways are 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 had abscesses in the lungs and on the body. Robins chronicles a patient with the longest known infection (15 years, only five of which were latent) who finally died of disseminated disease. Symptoms of this particular disseminated infection included nasal and aural discharge, submaxillary adenitis, nose phlegmon, nasal septum perforation, jaundice, diarrhea, and amyloid disease.^{4,7}

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 locations of infection. Before antibiotics, disseminated infection was ultimately fatal either by recurrence of acute disease or from chronic wasting. Based on the few cases treated with antibiotics, survival is likely if early and long-term effective therapy is instituted. Even with treatment, clinical symptoms may continue for several months before complete resolution, particularly if treatment is delayed.

Complete blood count and chemistry studies for glanders patients vary depending on the disease's location 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 (see Table 6-1). Neutropenia or neutrophilia, with or without a left shift, may be transient findings. Leucopenia with mild to moderate relative lymphocytosis was seen in three of the six laboratory-acquired infections reported by Howe and Miller,¹ 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 that the mortality rate for localized disease is 20% when treated, and the overall mortality rate is 40%.³⁸ 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 patients (37%) experiencing a delay in effective treatment of 2 months. Even 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 patients who received delayed treatment still recovered with only 10 days of sulfadiazine, although 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.

Diagnosis

Definitive diagnosis of glanders is by isolation and positive identification of the organism. Physical findings that support the differential diagnosis of glanders may be linked to the potential route of infection. With pulmonary involvement—likely from aerosol exposure—suspect clinical signs and symptoms include oropharyngeal injection, headache, chest pain, fever, rigors, night sweats, fatigue, cough, nasal discharge, and diagnostic imaging studies that support localized or lobar pneumonia, bronchopneumonia, miliary nodules, lobar infiltrative pneumonia, and consolidation (early) or cavitating (later) pulmonary lesions (see Table 6-1). Neurologic signs may also be present, with or without obvious pulmonary signs. With cutaneous involvement and regional lymphadenopathy likely from percutaneous exposure to infected equids or contaminated fomites, clinical signs and symptoms include lymphadenopathy with or without ulceration and single or multiple cutaneous eruptions that may heal slowly, particularly along lymphatic pathways (see Patient 8, Table 6-1). For presentation at autopsy, suspect findings include disseminated nodular and ulcerative disease, particularly involving the spleen, lungs, and liver. Cultures of nodules in septicemic cases usually establish the presence of *B mallei*. These presentations support glanders as a differential diagnosis and prompt further testing to rule out *B mallei* infection.

The development of adequate diagnostic tests that could identify infected animals, particularly those that were asymptomatic, finally allowed glanders control through test and slaughter programs. Until this breakthrough, isolating the agent, particularly from chronically infected animals, was difficult. A potential glanders clinical presentation in a human patient should prompt immediate notification of local animal health authorities to explore potential cases of glanders in livestock, particularly equids. The converse is also true; glanders as a potential differential

diagnosis in livestock warrants immediate notification of local regulatory animal and public health authorities. Cutaneous ulcerative disease outbreaks in sheep, goats, and swine accompanying suspected human cases would be more consistent with a *B pseudomallei* (melioidosis) outbreak than with *B mallei*. Because of the rarity of natural glanders infection, bioterrorism should also be immediately suspected, particularly in regions where glanders has been eradicated. Human glanders without animal exposure or more than one human case is presumptive evidence of a biowarfare attack. With this suspicion, regional public health authorities can initiate an appropriate emergency public health response for disease prevention, environmental decontamination, epidemiological investigation, and criminal investigation.^{23,48}

Because *B mallei* has a high potential for aerosol or droplet production and laboratory-acquired infection, BSL-3 personnel and primary containment precautions are indicated for activities attempting to rule out *B mallei* infection. Aseptically collected exudates from abscesses, cutaneous and mucous membrane lesions, sputum, and blood as well as aspirates from preerupting nodules and abscesses are excellent culture sources. Blood cultures are often not productive unless disease stage is near terminal.⁴⁹ Bacteremia is more likely during febrile peaks (and acute disease), thus sampling during such peaks may enhance chances for a productive culture. Among the eight US laboratory-acquired infections, blood cultures were attempted at least once within several weeks of initial presentation. In at least the first seven cases, special media were used to enhance growth of *B mallei*. All were negative (see Table 6-1). In the eighth case, a positive blood culture was obtained 2 months after initial presentation during an acute septicemic relapse in which the patient was in a guarded condition.⁵⁰

Growth and Morphology

In endemic regions, biochemical assays and observation of colony and cell morphology may still be a practical means to definitively diagnose glanders. These methods may take 2 to 7 days to confirm a diagnosis.⁵¹ Gram stains of pus from lesions may be productive, but microorganisms are generally difficult to find, even in acute abscesses.⁴⁹ *B mallei* can be cultured and identified with standard bacteriological media. In potentially contaminated samples, supplements to inhibit the growth of gram-positive organisms (eg, crystal violet, proflavine, penicillin) or *B mallei*-selective media may be useful.^{52,53} Optimum growth temperature is approximately 37°C.⁴⁷ Growth is typically slow on nutrient agar, but is rapid (2 days) when

enhanced with 1% to 5% glucose and/or glycerol, and on most meat infusion nutrient media.^{52,54} *B mallei* colonies typically are about 1 mm in width, white (turning yellow with age), and semitranslucent and viscid on Loeffler's serum agar and blood agar. Colonies have a clear honey-like layer by day three, later darkening to brown or reddish-brown when grown on glycerin-potato medium. Selective inhibition of *B pseudomallei* and *Pseudomonas aeruginosa* growth may be enhanced by noting the following: *B mallei* does not grow at 42°C; *B pseudomallei* and *P aeruginosa* do. Nor does *B mallei* grow at 21°C; *P aeruginosa* does. Furthermore, *B mallei* does not grow in 2% sodium chloride solution, nor on MacConkey agar; both *B pseudomallei* and *P aeruginosa* do.⁶

B mallei is a small, nonmotile, nonsporulating, nonencapsulating aerobic gram-negative bacillus approximately 2 to 4 µm long and 0.5 to 1 µm wide (Figure 6-2). *B mallei* is facultatively anaerobic in the presence of nitrate.^{47,55} 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

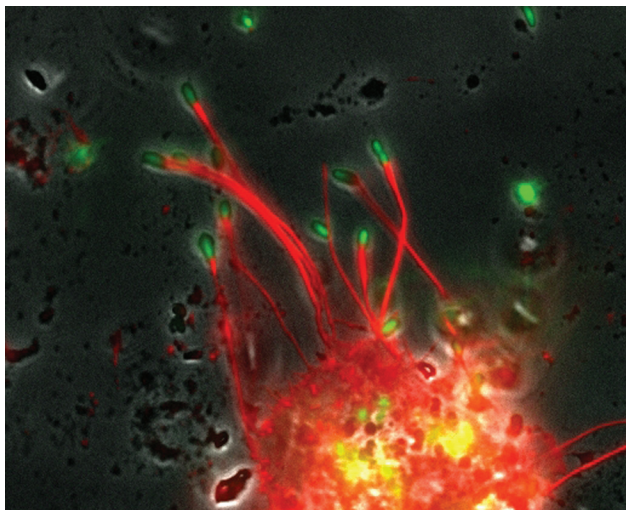


Fig. 6-2. The *B mallei* ATCC 23344 animal pathogen-like type 3 secretion system is involved in the induction of actin-based host cell membrane protrusions. J774.2 cells were infected with wild-type *B mallei* expressing green fluorescent protein at a multiplicity of infection of 10 bacteria to 1 macrophage. At 6 hours postexposure, cells were fixed and cellular actin was stained with Alexa Fluor⁵⁶⁸ phalloidin and viewed at a magnification x 630.

Photograph: Courtesy of Dr Ricky Ulrich, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

may be pleomorphic.⁵² In vivo, *B mallei* is found most often to be extracellular. Samples should be designated as “glanders suspect” because of the rarity of disease. Sample security, including appropriate chain of custody documentation, is also prudent for all samples. Automated bacterial identification systems may misidentify the organism. In the eighth US laboratory-acquired infection, such an automated system identified the agent as *Pseudomonas fluorescens* or *P putida*.⁵⁰ *B mallei* may have a beaded appearance in histopathology sections, where organisms tend to be difficult to demonstrate.³⁴

Isolation

Animal inoculation studies have been used to isolate the organism, but such studies may be impractical now for two reasons: (1) the time required for disease to manifest, and (2) logistical requirement for special containment facilities. Intraperitoneal inoculation of suspect *B mallei* exudate into intact male guinea pigs was once popular because they are nearly universally susceptible to infection and tend to produce a well-described localized peritonitis and associated orchitis. Loeffler first described this consistent experimental syndrome in 1886,² and it later was called the Strauss reaction.^{22,54} Although this method of testing is sensitive, the clinical course runs nearly a month, which precludes rapid diagnosis.² Because *B mallei*, *B pseudomallei*, and *P aeruginosa* also produce identical clinical signs in intact male guinea pigs,⁶ positive identification of the organism from the testes is still required to enhance sensitivity.

The field mouse (*Arvicola arvalis*) was also considered as a potential host for inoculation and isolation because of extremely high susceptibility to infection (even more so than the donkey) and predictable short disease course ending with sudden death in 3 to 4 days.² Upon necropsy, generalized subcutaneous infiltrate extending into superficial musculature, lymphangitis and lymphadenitis, enlarged spleen, liver infiltration, normal kidneys, and normal testicles are consistent findings in field mice. However, if exudates with mixed bacterial flora (which may be common with nasal exudates and sputum) are used in field mice, organisms causing other bacterial disease may competitively exclude expression of glanders disease.² In the seventh US laboratory-acquired infection, two mice injected with the patient's sputum died within 24 hours. From peritoneal washings taken from the mice, gram-positive cocci in pairs typed as pneumococci were readily observed, as were occasional gram-negative rods found to be “*Malleomyces mallei*” (name for *B mallei* at the time).

Organism Identification

The *B mallei* genome has been sequenced (see the Institute for Genomic Research Web site, www.tigr.org),⁵⁶ which results in an enhanced ability to specifically identify this microorganism and further demonstrate how *B mallei* interacts with its host. Several relatively new molecular-method diagnostic capabilities exist to reliably confirm specific identification of *B mallei* within several hours, including polymerase chain reaction-based assays and DNA gene sequencing.⁵⁷⁻⁵⁹ The latter methods, as phenotypic testing and 16S ribosomal RNA gene-sequence analysis, identified *B mallei* from other *Burkholderia* species in the 2000 US laboratory-acquired infections.⁵⁰ A polymerase chain reaction procedure based on differences detected in ribosomal DNA sequences was also developed to distinguish *B mallei* from *B pseudomallei*.⁵⁷

Polymerase chain reaction-based techniques and DNA gene sequencing are increasingly used in clinical settings and public health laboratories for bacterial identification.⁶⁰ Automation of sequencing and improved efficiencies of reagents have 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.⁵⁷ These methods are not yet widely available for *B mallei* identification; however, the current interest in biowarfare defense research is prompting a continued increased capability based on recent publications.^{57-59,61,62}

Pulsed-field gel electrophoresis and ribotyping have been used to identify strains of *B pseudomallei* in outbreaks.⁶³ These methods have also been used to differentiate pathogenic *B pseudomallei* strains from less virulent strains.⁶⁴ Pulsed-field gel electrophoresis and ribotyping may be as useful for identification and virulence testing of *B mallei*, although these methods may be more labor intensive and time consuming than gene sequencing. Gas liquid chromatography of cellular fatty acids was used to help identify the organism as a *Burkholderia* genus in the laboratory-acquired infection in 2000.

Imaging Studies

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. Pulmonary abscesses, which may be single or multiple, undergo central degeneration and necrosis, which radiographi-

cally resemble cavitation. Unilateral or bilateral bronchopneumonia and a smattering of miliary nodules may be seen. Because of the potential for disseminated disease, computed tomography imaging is useful for monitoring deep tissues and visceral organs.

Serology and Mallein Testing

There are no specific serologic tests for human glanders diagnosis. The agglutinin test, complement fixation test (CFT), and mallein testing are not consistent in humans, nor are they particularly timely. The indirect hemagglutination and CFTs have been tried,^{65,66} but the CFT may not detect chronic cases of glanders.⁴² Serologic tests were instrumental, however, in diagnosing all seven US laboratory-acquired infections between 1944 and 1953 (see Table 6-1). Although sensitive, agglutinin tests may be difficult to interpret because of potentially high background titers of up to at least 1:320. Titers rising from 0 to 320 may be significant, however, as was the case with patient 6 (see Table 6-1). For at least four of the seven aforementioned cases, agglutinin titers developed in 3 weeks from disease onset (see Table 6-1). The CFT was initially used in the diagnosis of glanders in 1909⁶⁷ shortly after the mallein test was developed. The CFT is still used for glanders screening in animals in the United States; mallein testing is used only in animals positive for complement fixation antibodies.³⁹ The CFT is believed to be more specific than the agglutinin test; a positive titer is considered to be $\geq 1:20$. In at least one patient (patient 6), however, the CFT was persistently negative. Patient 5 was also persistently negative but may not have been tested for a 70-day interval between the 17th and 87th day after disease onset; the agglutinin test was diagnostic by the 22nd day.

The US Army Medical Research Institute of Infectious Diseases has developed an enzyme-linked immunosorbent assay (ELISA) for human glanders. In laboratory testing, an ELISA could differentiate serum from a glanders patient from sera from patients with clinical cases of anthrax, brucellosis, tularemia, Q fever, and spotted fever.⁶⁸ However, an ELISA cannot distinguish glanders from melioidosis, caused by *B pseudomallei*, a closely related microorganism.

Development of a human mallein skin test was attempted, but delay of up to several weeks postinfection for positive result rendered it of little diagnostic value.⁶⁹ Modified equine mallein tests have infrequently been used in humans, however.¹³ At the station hospital at Camp Detrick, 0.1 mL of 1:10,000 diluted commercial mallein was injected intradermally into the forearm, and the test was read at 24 and 48 hours. Five of the first seven patients tested positive as early as the 18th

day of disease. In one patient (patient 4), the modified mallein test was the first of the three tests to show positive results (see Table 6-1). In contrast, patient 5 did not test positive until the 72nd day postdisease onset, whereas agglutinin was positive by day 22. The CFT, agglutinin titer, and mallein tests remained positive for no less than 10 months in the two patients (patient 1 and patient 2) whose diagnoses were delayed and who received the shortest course of antibiotics. Both responded quickly to treatment, however. Patient 3 also had persistently positive serology and a protracted illness. Serology may be useful to monitor cure post-treatment, if not for initial diagnosis.

Diagnosis in Equids

Whether naturally occurring or related to bioterrorism, a suspected case of human glanders warrants the investigation of potential contact equids or fomites. Physical findings in equids that support the differential diagnosis of glanders include fever; white-to-greenish viscous unilateral or bilateral nasal exudate that dries, forming thin yellowish crusts along the external nares; irregularly shaped abscesses on the nasal septum; regional lymphadenopathy; boil-like lesions with thick, ropy lymphatic pathways tracking from them; swelling of the limbs; dull hair coat; cough; weakness; and emaciation. Universal precautions are warranted when handling animals or fomites suspected or known to be infected. Because glanders may be latent or clinically inapparent, potential contacts to a human (or livestock) case should undergo systematic testing to help identify a potential outbreak.^{18,20,26,44}

In the United States glanders has been considered a foreign animal disease (FAD) since its eradication in 1942. US Department of Agriculture (USDA) veterinarians are trained to recognize and control FADs—including glanders—and help mitigate the shortfall created by the unfamiliarity with glanders in human patient care settings. In the United States the USDA and the Department of Homeland Security have elements of regulatory authority for unintentional FAD outbreaks. When a FAD or other federally regulated disease is suspected in the United States, an emergency response system is activated. Where intentional transmission is suspected, the Federal Bureau of Investigation should be contacted immediately, and it will take the lead in the investigation. Many other countries have a corresponding FAD (includes glanders) emergency response system. Therefore, human patient care and public health systems around the globe should partner with local and regional animal health authorities when there is any suspicion of zoonotic disease.

The OIE provides technical support to member countries that request assistance with animal disease control and eradication operations, including zoonoses. The OIE also publishes the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*, a compilation of diagnostic procedures and a useful reference for any diagnostic laboratory, to coordinate methods for the surveillance and control of the most important zoonotic and animal diseases, including glanders.⁵⁴ The manual includes standards for the most current laboratory and diagnostic tests, and the production and control of biological products for veterinary use around the world.

For any case in which glanders must be ruled out in livestock, regionally assigned veterinarians respond after notification to quickly identify, contain, diagnose, and eradicate glanders from livestock in accordance with local or regional animal and public health authorities. The veterinarians also work with regional veterinary reference laboratories to ensure diagnostic samples are harvested and submitted accordingly.

Aseptic collection of specimens and laboratory-handling procedures are similar to those described for humans. *B mallei* may be isolated from fresh cutaneous lesions, blood (when pyrexia), nasal exudate, and various lesions at necropsy. Several tests are available for regulatory veterinarians to help diagnose glanders in equids. The CFT, indirect hemagglutination test, and ELISA are among the most highly sensitive tests for glanders in equids. The CFT is reported to be 90% to 95% sensitive with the ability to detect positive sera as soon as 1 week after infection. In chronic cases, sera are typically positive for a long time.²⁰ A limitation to the CFT is that a large percentage of donkey, mule, and pregnant mare sera are anticomplementary and cannot be effectively tested.⁵⁴ Counter immunoelectrophoresis⁷⁰ and immunofluorescence tests as well as agglutination and precipitin tests are available, although the latter two are unreliable for horses with chronic glanders and animals in poor condition. An immunoblot method has also been developed.⁷¹

A recently developed dot ELISA that rivals other tests economically was found to be the most sensitive compared to CFTs, indirect hemagglutination, and counter immunoelectrophoresis tests, and it is faster and easier to administer. The dot ELISA is not influenced by potential anticomplementary activity of some sera or other spurious activity that can be associated with the CFT.⁷² The test is named for the positive reaction that is indicated by the appearance of a clearly visible brown dot in the antigen-coated area. Mallein testing within 6 weeks interferes with test results, however. Thus, dot ELISA subsequent to mallein testing must be delayed. Low antibody levels of $\leq 1:100$ can be demonstrated in the normal equine population. Natural infection and

sensitized equids (eg, from mallein) have dot ELISA titers that range from 1:400 to 1:25,600.⁷² Positive dot ELISA titers may be seen 4 days postinfection, are present by 6 days, and persist for at least 7 weeks. All serologic tests for glanders in equids cross-react with those for *B pseudomallei*, which causes melioidosis. Thus, where melioidosis is endemic, serologic testing may result in false positive results.²²

The mallein test was the first diagnostic test for glanders and has been the bastion of field diagnosis and eradication programs since the 1890s. Russian military veterinarians Gelman and Kalning first developed the test in 1891,^{4,6} and the United States and Canada began using it as a diagnostic tool in 1905.²⁰ Originally cultured for 4 to 8 months, mallein is a heat-treated lysate of *B mallei* containing both endotoxins and exotoxins produced by the organism. The test works similarly to tuberculin testing. Glanders-infected animals become hypersensitive to mallein, exhibiting local pain and swelling, as well as a systemic reaction including a marked temperature increase, after inoculation. After confirmation of normal body temperature, mallein is injected intradermally either into the lower palpebrum (intradermo-palpebral test) or subcutaneously in the neck region (subcutaneous test). A third and slightly less reliable procedure is to instill a few drops of mallein onto the eye near the medial canthus (ophthalmic test). The intradermo-palpebral test is preferred.⁷³ Subsequent monitoring of the animal and interpretation of positive results depend on the method of administration and should be done by the animal health authorities who administered the test. In advanced clinical disease in horses and acute infection in donkeys and mules, however, mallein testing may give inconclusive results.⁷⁴ Also, testing of chronically infected or debilitated equids may give negative or inconclusive results. In either case additional testing methods are required. Mallein testing (inoculation) may trigger a humoral response and subsequent serologic reaction to the CFT, particularly when administered subcutaneously. Although thought to be transient, this seroconversion may become permanent after repeated mallein testing, which is an important consideration for equids that may be exported to regions that depend on the CFT.

Treatment

Because human glanders cases are rare, limited information exists regarding antibiotic treatment for humans. *B mallei* infection responds to antibiotic therapy; however, recovery may be slow after a delayed diagnosis or with disseminated disease. The scientific literature reports that *B mallei* is susceptible to the following antibiotics in vitro:

- amikacin,
- netilmicin,
- gentamicin,
- streptomycin,
- tobramycin,
- azithromycin,
- novobiocin,
- piperacillin,
- imipenem,
- ceftazidime,
- tetracycline,
- oxytetracycline,
- minocycline,
- doxycycline,
- ciprofloxacin,
- norfloxacin,
- ofloxacin,
- erythromycin,
- sulfadiazine, and
- amoxicillin-clavulanate.⁷⁵⁻⁸²

Aminoglycosides and other antibiotics incapable of penetrating host cells are probably not useful in vivo because *B mallei* is a facultative intracellular pathogen.^{79,80,82} Susceptibility to streptomycin and chloramphenicol in vitro has been inconsistent, with some researchers reporting sensitivity and others reporting resistance.^{6,78,80}

Most *B mallei* strains exhibit resistance to the following antibiotics:

- amoxicillin,
- ampicillin,
- penicillin G,
- bacitracin,
- chloromycetin,
- carbenicillin,
- oxacillin,
- cephalothin,
- cephalixin,
- cefotetan,
- cefuroxime,
- cefazolin,
- ceftriaxone,
- metronidazole, and
- polymyxin B.^{6,11,25}

Antibiotics have been tested against glanders in equids, hamsters, guinea pigs, and monkeys.^{77,81-85} Sodium sulfadiazine—but not penicillin or streptomycin—was effective for treating acute glanders in hamsters.⁸¹ Doxycycline and ciprofloxacin were also examined in the hamster model of glanders.⁸² Doxycycline therapy was superior to ciprofloxacin therapy, but some of the

treated animals relapsed in 4 to 5 weeks after challenge. Hamsters were also infected subcutaneously or by aerosol with *B mallei* and were treated with ofloxacin, bisepitol, doxycycline, and minocycline.⁸³ Although all of the antibiotics exhibited some activity in animals challenged subcutaneously, 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.⁸³

The majority of human glanders cases occurred before antibiotics, and over 90% of these people died.⁸⁶ Several human glanders cases have been recorded since the 1940s—primarily in laboratory workers—and these have been successfully treated with antibiotics.^{1,50,87,88} Sulfadiazine was used successfully in the first six US laboratory-acquired infections.¹ The seventh case was successfully treated with the tetracycline compound aureomycin. Two additional cases were successfully treated with sulfadiazine in 1949 and 1950.⁸⁷ Disseminated glanders in a stable hand who had only indirect contact with horses was also successfully treated with aureomycin in Austria in 1951.²⁹ Streptomycin was used to treat a patient infected with *B mallei* and *Mycobacterium tuberculosis*.⁸⁸ Treatment with streptomycin reportedly cured the glanders, but had little effect on the tuberculosis of this patient's bone. 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.⁵⁰ Susceptibility testing of the *B mallei* isolate in this case demonstrated sensitivity to the former two drugs.⁵⁰ A 6-month course of doxycycline and azithromycin followed, although retrospective susceptibility testing found that the organism was resistant to azithromycin. Diagnostic imaging of the patient's splenic and hepatic abscesses through the 6-month course showed their near complete resolution.

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 and trimethoprim-sulfamethoxazole are recommended for at least 20 weeks plus oral chloramphenicol for the first 8 weeks.²⁴ For severe disease, either ceftazidime at 40 mg/kg intravenously (IV) every 8 hours, or imipenem IV at 15 mg/kg every 6 hours (maximum 6 g/day), or meropenem at 25 mg/kg IV every 8 hours (maximum 6 g/day) and trimethoprim-sulfamethoxazole at 8 mg trimethoprim/kg per day IV in four divided doses is recommended. IV therapy should be continued for at least 14 days and until the patient is clinically improved. Oral maintenance therapy for mild disease can be continued from that point.²⁴

Patients with the mildest of systemic symptoms should consider combined therapy for at least the first month. For visceral and severe disease, prolonged treatment for up to a year is recommended. Abscesses may be surgically drained, depending on their location.³⁸ For infections that are slow to clear, long-term follow-up and possibly prolonged tailored therapy is recommended because of the intractable nature of glanders. Patients should be followed at regular intervals for at least 5 years after recovery. Diagnostic imaging is useful to follow the reduction and recurrence of abscesses, serology may help to monitor the clearing of antibody, and inflammatory markers may also suggest recurrence of a latent infection. Patients should be informed of the life-long risk of relapse and advised to alert their healthcare providers of their previous history, particularly if they develop a febrile illness. These actions are especially important if the patient might have been infected with a genetically engineered strain of *B mallei*.

Prophylaxis

There is no evidence that previous infection or vaccination provides immunity against glanders.^{6,89} Infections in horses that seemed to symptomatically recover from glanders have recrudesced when the animals were challenged with *B mallei*. Inoculating *B mallei* into chronically infected horses generally produced at least local infections and occasionally a manifestation of classic glanders. Numerous attempts to vaccinate horses and laboratory animals against glanders were unsuccessful between 1895 and 1928. For most chronically infected horses, experimental vaccination did not change the course of their illness. Vaccines were made by treating bacterial cells with urea or glycerin⁶ or by drying the glanders bacilli.⁸⁹ Experiments on protective immunity in horses have given ambiguous results.^{2,6} Passive immunity experimentation using equine sera has also failed.⁶ A nonviable *B mallei* cellular vaccine failed to protect mice from a parenteral live challenge.⁹⁰ This vaccine stimulated a mixed T-cell helper (Th)1- and Th2-like immune response. This study suggested that nonviable *B mallei* cell preparations may not protect mice because of the failure to induce a strong Th1-like immune response. Because no vaccines protected animals from disease, control and eradication of glanders were dependent on eliminating infected horses and preventing them from entering glanders-free stables.

Protective immunity in humans after infection 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. Mendelson reported guarded postvaccination success in a young person with severe ocular and oro-nasal involvement.³⁰ Thus, patients who recover

may still be susceptible, which makes reuse of the agent in biowarfare necessary to consider.

Although unsuccessful attempts to develop a glanders vaccine were initiated over 100 years ago, using modern approaches to identify virulence factors and studying the ways putative vaccines modulate the immune system could possibly result in the development of a vaccine to induce sterile immunity. The initial attempts to protect mice against an aerosol-acquired infection using an irradiation-killed *B mallei* cellular vaccine resulted in an increased time to death, compared to controls, but spleens of survivors were not sterile.⁹¹ The most desirable glanders vaccine would be a recombinant protein or a biochemically purified preparation that provides long-term sterile immunity.

Antibiotics may offer some protection, however, against a *B mallei* strategic attack. Prophylaxis with doxycycline and ciprofloxacin given before and coincident with intraperitoneal inoculation in rodents caused the minimum lethal dose to rise several thousand-fold, but did not completely protect against infection.⁸² This approach is limited by the possibility that the biological agent may be engineered to resist the anticipated antibiotic regimen (as is true for other types of biowarfare).

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, patient care personnel, public health officials, and local veterinarians should investigate any potential exposure to infected equids. 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 case of deliberate release of *B mallei*, emergency response personnel entering a potentially heavily contaminated area should wear protective gear, including a mask with a biological filter. 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 according to local protocol. All waste should be managed according to 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 probably low.⁴⁸ Prophylactic treatment with ciprofloxacin or doxycycline may help to prevent infection in those potentially exposed, including emergency responders.

Environmental contamination declines after sunlight exposure and drying. Monitoring highly contaminated areas is indicated, however, and seeking the advice of FAD experts is recommended. *B mallei* can remain viable in tap water for at least 1 month²⁰ and 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, at least 5% calcium hypochlorite, 70% ethanol, 2% glutaraldehyde, iodine, benzalkonium chloride, at least 1% potassium permanganate, at least 3% solution of alkali, and 3% sulfur-carbolic solution. Phenolic and mercuric chloride disinfectants are not recommended.^{6,22}

Because human-to-human transmission has occurred nosocomially and with close personal contact, standard precautions are recommended, including 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 should be used in the laboratory. Appropriate barriers to direct skin contact with the organisms are mandatory.^{92,93} Family contacts should be advised of blood and body fluid precautions for patients recovering at home. Barriers protecting mucous 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.¹⁸ 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

Glanders is a Category B disease of concern for bioterrorism by the Centers for Disease Control and Prevention because the agent is believed to be moderately easy to disseminate. Dissemination would

result in moderate morbidity and low mortality, and enhancements to current diagnostic capabilities and disease surveillance would be required to rapidly and accurately diagnose the disease.

Because *B mallei* is a contender for use as a biological warfare or terrorism agent, the clinical index of suspicion should increase for glanders disease in humans. The rarity of recent human cases may make glanders a difficult diagnosis even in regions with exceptional medical facilities. As is the case with many rare diseases, final diagnosis and appropriate treatment are often delayed, sometimes with disastrous results. Without a higher index of suspicion, diagnostic laboratories might not conduct tests appropriate to detect *B mallei*, which happened in 2000 in the eighth US laboratory-acquired infection case.⁵⁰

Further studies are needed to fully assess the usefulness of 16S rRNA sequencing in epidemiological investigations and the potential of using the subtle variations in the 16S rRNA gene sequence as a subtyping method for virulence and toxin production.

The genetic homology between *B mallei* and *B pseudomallei* may cause confusion in identifying the infectious agent, especially in areas endemic for *B pseudomallei*, which presents another challenge and invites further research. The capability to distinguish

virulent strains from nonvirulent naturally occurring strains would also be useful. Finally, more research on antibiotic susceptibilities to *B mallei* is also warranted. Specifically, studies to consider an aerosol threat from a virulent strain and to distinguish the effectiveness of therapeutic agents for treating septicemic and pulmonary infections are indicated. The potential for prophylactic treatment regimens should also be investigated.

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. Therefore, 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 USDA, the Centers for Disease Control and Prevention, and the Department of Health and Human Services to control and eradicate the disease.

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Chapter 7

MELIOIDOSIS

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INTRODUCTION

INFECTIOUS AGENT

MILITARY RELEVANCE

DISEASE

- Epidemiology
- Pathogenesis
- Clinical Disease
- Diagnosis
- Treatment
- Prevention

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 Whitmore and Krishnaswami isolated a gram-negative bacillus that resembled the glanders bacillus, *Bacillus mallei*, from postmortem tissue samples.³ However, the new bacillus could be differentiated from *B mallei* by its motility, luxuriant growth on peptone agar, and wrinkled colony morphology; it was subsequently named *Bacillus pseudomallei*.^{3,4} Whitmore's detailed account of the first 38 human cases of this disease demonstrated that most of those affected were morphine injectors who 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 Stanton and Fletcher isolated an infectious agent from diseased animals that was indistinguishable from Whitmore's bacillus, and they named it "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.⁸

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; it 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 attributable to a variety of pathogens, resulting in the nickname "the remarkable imitator."¹² The majority of melioidosis cases have identified risk factors, including diabetes, alcoholism, chronic renal disease, cystic fibrosis, and steroid abuse.¹³ AIDS does not seem to be a major risk factor for melioidosis. Healthy individuals can also contract melioidosis, 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.^{11,13-27}

INFECTIOUS AGENT

The bacterium that causes melioidosis, now designated *Burkholderia pseudomallei*,²⁸ has undergone numerous name changes since its original classification as *B pseudomallei*, including (a) *Bacterium whitmori*, (b) *Bacillus whitmori*, (c) *Pfeifferella whitmori*, (d) *Pfeifferella pseudomallei*, (e) *Actinobacillus pseudomallei*, (f) *Lofflerella whitmori*, (g) *Flavobacterium pseudomallei*, (h) *Malleomyces pseudomallei*, and (i) *Pseudomonas pseudomallei*. The nonsporulating, gram-negative bacillus is an environmental saprophyte found in surface waters and wet soils in endemic regions.²⁹⁻³⁶ Individual cells, which are approximately 0.8 × 1.5 μm, have a polar tuft of two to four flagella and exhibit bipolar staining with a "safety pin" appearance.^{37,38} *B pseudomallei* is metabolically versatile and can grow on numerous carbon sources.^{28,39} 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.^{10,40,41} The optimal survival temperature for *B pseudomallei* is between 24°C and 32°C, but it can grow at temperatures up to 42°C.^{42,43} *B pseudomallei* demonstrates considerable interstrain and medium-dependent colony morphology.⁴⁴⁻⁴⁶ 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.⁴⁷ Two distinct colony phenotypes are commonly observed on this medium (Figure 7-1), probably because of the differential uptake of crystal violet and neutral red or the differential production of ammonia and oxalic acid.^{47,48} Most strains appear lavender after 2 to 3 days of incubation at 37°C, but some isolates appear deep purple (see Figure 7-1). After 5 days at 37°C, the colonies often become dull and wrinkled (see Figure 7-1) and emit a distinctive sweet earthy smell. Other selective media have also been used to isolate *B pseudomallei* from contaminated specimens.^{49,50}

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 recently determined.⁵¹ The 7.25-megabase pair (Mb) genome was composed of two circular replicons, termed chromosome 1 (4.07 Mb) and chromosome 2 (3.17 Mb). The G + C content of the genome was 68% and 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); and chromosome 2 encoded a high proportion of accessory functions (adaptation to atypical conditions, osmotic protection, and secondary

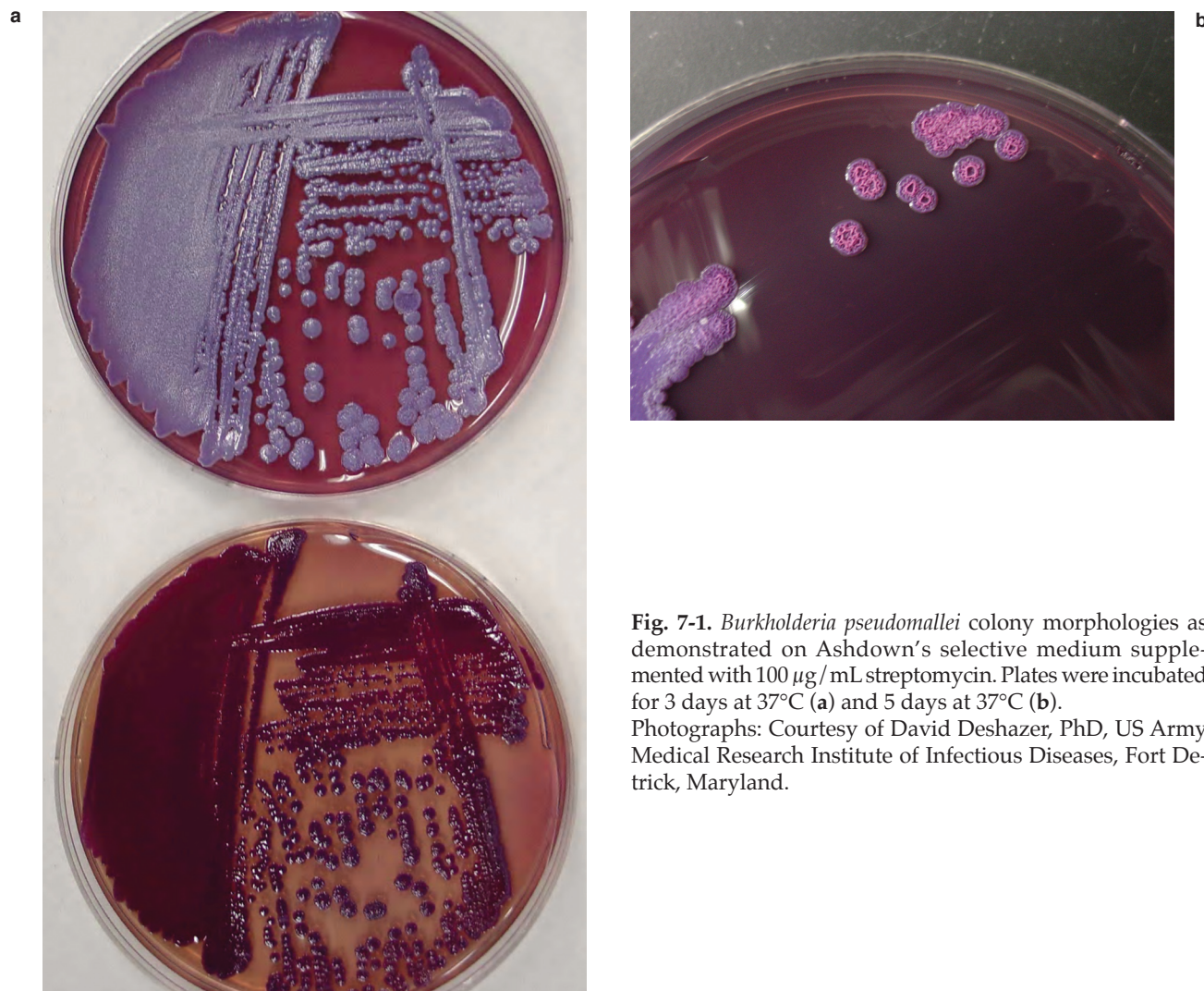


Fig. 7-1. *Burkholderia pseudomallei* colony morphologies as demonstrated on Ashdown's selective medium supplemented with 100 $\mu\text{g}/\text{mL}$ streptomycin. Plates were incubated for 3 days at 37°C (a) and 5 days at 37°C (b). Photographs: Courtesy of David Deshazer, PhD, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

metabolism).⁵¹ 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 tRNA 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.⁵¹ 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 caused by laterally acquired mobile genetic elements.⁵¹⁻⁵⁶ These genomic islands may provide strains that harbor a metabolic and/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.^{27,57-59} Recently, the draft genome sequences of an additional nine *B pseudomallei* isolates (1710a, 1710b, 406e, 1106a, 1106b, S13, Pasteur 52237, 668, and 1655) were determined and deposited in Genbank, dramatically enhancing the amount and diversity of genome sequence data available for the study of *B pseudomallei*.

MILITARY RELEVANCE

Throughout the 20th century, melioidosis had an impact on the health of soldiers serving in Asia during times of war and peace.⁶⁰ Sporadic melioidosis

infections occurred in US and Japanese soldiers during World War II,^{38,61,62} and recrudescence melioidosis cases in World War II veterans were also reported.^{63,64}

During the French Indochina War (1946–1954), there were at least 100 melioidosis cases among French forces during their fight against the resistance movement led by the Viet Minh.^{19,60} Fewer than 300 melioidosis cases occurred among US soldiers during the Vietnam War,¹⁹ and additional cases did not surface until years after the war's end, leading to the nickname "Vietnam Time Bomb."⁶⁵⁻⁶⁷ Twenty-three melioidosis cases were reported in the Singapore armed forces from 1987 to 1994.⁶⁸ The infection rate in these relatively healthy servicemen was approximately 4-fold higher than the rate in Singapore's general population, 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 Category B biological terrorism agent that must be handled in biosafety level 3 laboratories.⁹ Biosafety level 3 facilities incorporate specialized negative-air pressure ventilation systems, well-defined

biosafety containment equipment, and protocols to study agents that can be transmitted through the air and cause potentially lethal infection. Category B agents have the potential for large-scale dissemination with resultant illness and death, but generally would be expected to have lower medical and public health impact than Category A agents.⁹ *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.⁶⁹⁻⁷¹ However, *B mallei* was used as a biological warfare agent during the American Civil War, World War I, World War II, and in Afghanistan between 1982 and 1984.^{2,70,72,73} The usefulness of *B pseudomallei* as a biological warfare agent is 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.

DISEASE

Epidemiology

Melioidosis cases have been increasingly 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.^{11,13,20} Melioidosis has also been observed in the South Pacific, Africa, India, and the Middle East. In addition, sporadic melioidosis cases have occurred in the Western Hemisphere in Aruba, Brazil, Mexico, Panama, Ecuador, Haiti, Peru, and Guyana.^{11,13,20} In endemic regions, the disease occurs in humans, sheep, goats, horses, swine, cattle, dogs, cats, and other animals.^{15,24} Melioidosis cases that occur in temperate regions often result from recent travel to endemic areas.^{18,74-77}

Pathogenesis

Several animal models of melioidosis have been developed to study pathogenesis, virulence factors, and efficacy of antibiotics and vaccines.⁷⁸⁻⁸⁶ In general, hamsters and ferrets are highly susceptible to experimental melioidosis (median lethal dose [LD₅₀] of < 10² bacteria), and rats, pigs, and rhesus monkeys are relatively resistant (LD₅₀ of > 10⁶ bacteria). Infant rats can be made more susceptible to infection by intraperitoneal injection of streptozotocin, a compound that induces diabetes.^{82,87} The LD₅₀ of *B pseudomallei* for nondiabetic infant rats is greater than 10⁸ bacteria in streptozotocin-induced diabetic infant rats; the LD₅₀ is approximately 10⁴ bacteria. Mice and guinea pigs

exhibit intermediate susceptibility to experimental infection with *B pseudomallei*, but the LD₅₀ for mice varies widely depending on the route of infection, mouse strain, and bacterial strain.^{80,81,84,88}

Basic research on this pathogen has progressed rapidly over the past 5 years because of fears that *B pseudomallei* may be used as a biological weapon. The identification of virulence factors has been facilitated by the availability of genomic sequence data⁵¹ and the existence of a nonpathogenic *B pseudomallei*-like species designated *B thailandensis*.⁸⁹⁻⁹¹ *B pseudomallei* and *B thailandensis* strains are genetically and immunologically similar to one another, but *B thailandensis* is avirulent in animal models of infection and rarely causes disease in humans. 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.^{53,92,93} Exhibit 7-1 provides a brief description of all known *B pseudomallei* virulence factors, their mechanisms of action, and their relative importance in animal models of melioidosis.

B pseudomallei is a facultative intracellular pathogen that can replicate and survive in phagocytic and nonphagocytic cell lines.⁹⁴⁻⁹⁹ After the initial phase of infection, researchers postulate that *B pseudomallei* can persist in a dormant stage in macrophages for months or years.⁹⁹ 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.

EXHIBIT 7-1

CANDIDATE VIRULENCE FACTORS OF *BURKHOLDERIA PSEUDOMALLEI*

Factor	Description
Capsule	A 200-kd group 3 capsular polysaccharide composed of a homopolymer of -3)-2-O-acetyl-6-deoxy-β-D-manno-heptopyranose-(1-. ¹ Capsule mutants are highly attenuated in hamsters and mice. ^{2,3} The capsule may contribute to survival in serum by reducing complement factor C3b deposition. ⁴
TTSS	<i>B pseudomallei</i> harbors three distinct TTSS loci: (1) TTSS1, (2) TTSS2, and (3) TTSS3. ⁵ The TTSS1 and TTSS2 loci are similar to TTSS genes of the plant pathogen <i>Ralstonia solanacearum</i> and are not necessary for virulence in hamsters. ⁵ The TTSS3 locus is similar to the TTSS in <i>Salmonella</i> and <i>Shigella</i> ⁶ and is required for full virulence of <i>B pseudomallei</i> in both hamsters and mice. ^{5,7} The effector proteins of TTSS3 facilitate the invasion of epithelial cells and escape from endocytic vesicles. ^{6,8}
Quorum sensing	<i>B pseudomallei</i> encodes three <i>luxI</i> homologues that produce at least three quorum-sensing molecules: (1) N-octanoyl-homoserine lactone (C8-HSL), ^{9,10} (2) N-decanoyl-homoserine lactone (C10-HSL), ^{9,11} and (3) N-(3-hydroxyoctanoyl)-L-homoserine lactone (3-hydroxy-C8-HSL). ⁹ It also has five <i>luxR</i> homologues to sense these signals. Mutations in all of the <i>luxI</i> and <i>luxR</i> homologues result in strains with decreased virulence in hamsters and mice, ^{9,11} but the virulence-associated genes regulated by this complex quorum-sensing system are under investigation.
LPS O-antigen	An unbranched heteropolymer with repeating D-glucose and L-talose units with the structure -3)-β-D-glucopyranose-(1-3)-6-deoxy-α-L-talopyranose-(1-. ¹²⁻¹⁴ LPS O-antigen mutants are attenuated in hamsters, guinea pigs, and infant diabetic rats and are killed by serum. ¹⁵ 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. ¹⁶
Flagellin	A surface-associated 43-kd protein that is required for motility. ^{17,18} Flagellin mutants are attenuated in mice, ¹⁹ but not in hamsters or infant diabetic rats. ¹⁸ Passive exposure studies demonstrated that flagellin-specific antiserum was capable of protecting infant diabetic rats from challenge with <i>B pseudomallei</i> . ¹⁷
Type II secretion	Required for the secretion of several exoproducts, including protease, lipase, and phospholipase C. ²⁰ The products secreted by this pathway appear to play a minor role in <i>B pseudomallei</i> pathogenesis. ²¹
Type IV pilin	<i>B pseudomallei</i> K96243 encodes four complete type IV pilin clusters. ²² A mutation in <i>pilA</i> , 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 <i>pilA</i> mutant was not attenuated in mice by the intraperitoneal challenge route, but was slightly attenuated by the intranasal challenge route. ²³
Biofilm formation	The extracellular slime matrix produced by <i>B pseudomallei</i> appears to be polysaccharide in nature, but the exact structure is unknown. ²⁴ Biofilm mutants were not attenuated in the mouse model of melioidosis, suggesting that the biofilm plays a relatively minor role, if any, in virulence. ²⁴
Malleobactin	A water-soluble siderophore of the hydroxamate class. ²⁵ The compound is capable of scavenging iron from both lactoferrin and transferrin in vitro. ²⁶ The genes encoding malleobactin biosynthesis and transport were recently identified, but malleobactin mutants were not tested in animal models of melioidosis. ²⁷
Rhamnolipid	A 762-Da glycolipid with the structure 2-O-α -L-rhamnopyranosyl-α -L-rhamnopyranosyl-β-hydroxytetradec anoyl-β-hydroxytetradecanoate (Rha-Rha-C14-C14). ²⁸ Rhamnolipid-treated cell lines exhibit profound morphological alterations, but the role of this glycolipid in virulence remains unknown. ²⁹
EPS	A linear unbranched polymer of repeating tetrasaccharide units composed of D-galactose and 3-deoxy-D-manno-octulosonic acid (KDO), with the following structure: -3)-2-O-Ac-β-D-Galp-(1-4)-α -D-Galp-(1-3)-β-D-Galp-(1-5)-β-D-KDOP-(2-. ³⁰⁻³² EPS is not produced by the closely related nonpathogenic species <i>B thailandensis</i> , suggesting that it may be a virulence determinant of <i>B pseudomallei</i> . EPS is probably produced during infection because sera from melioidosis patients contain IgG and IgM antibodies to EPS. ^{31,33}
Endotoxin	The lipid A portion of <i>B pseudomallei</i> LPS contains amide-linked 3-hydroxyhexadecanoic acids,

(Exhibit 7-1 continues)

Exhibit 7-1 continued

Actin-based motility	which are longer than the fatty acid chains of enterobacterial LPS. ³⁴ The endotoxic activity of <i>B pseudomallei</i> LPS was 10 to 100 times weaker than enterobacterial LPS in pyrogenic activity in rabbits, lethal toxicity in GalN-sensitized mice, and macrophage activation assays. However, the mitogenic activity of <i>B pseudomallei</i> LPS was much higher than enterobacterial LPS. ³⁴ The LD ₅₀ of purified <i>B pseudomallei</i> LPS in hamsters was 1,000 mg. ³⁵
Exotoxins	Once <i>B pseudomallei</i> 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. ³⁶⁻³⁸ The autotransported protein BimA is located at the pole of the bacterial cell and is responsible for the formation of actin tails. ³⁷ It is unknown if actin-based motility is required for virulence in animal models of melioidosis. There have been several reports in the literature about <i>B pseudomallei</i> exotoxins, ³⁹⁻⁴³ but the genes encoding these exotoxins have not been identified and no defined exotoxin mutants have been constructed. The role of exotoxins as <i>B pseudomallei</i> virulence factors is highly controversial, and there appears to be no correlation between in-vitro cytotoxicity and in-vivo virulence. ^{35,44} The K96243 genome sequence does not encode any homologues of known major toxins produced by other pathogenic bacteria. ²²

EPS: exopolysaccharide kd: kilodalton LPS: lipopolysaccharide TTSS: Type III secretion system

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(Exhibit 7-1 continues)

Exhibit 7-1 *continued*

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Clinical Disease

Melioidosis is a tropical bacterial disease with primary endemic foci in Southeast Asia, northern Australia, south Asia, and China. Hyper-endemic areas for melioidosis include northern Australia and northeastern Thailand, where the disease incidence peaks in the rainy season. Heavy rainfall probably results in a shift from percutaneous inoculation to inhalation as the primary mode of infection, which leads to more severe illness.¹⁰⁰ In these hyper-endemic areas, *B pseudomallei* causes a substantial burden of infectious disease. For example, at a northeast Thai hospital that serves nearly 2 million rural rice-farming families, nearly 20% of all community-acquired bacteremia that occurred during the rainy season resulted from *B pseudomallei*.¹⁰¹ Likewise, melioidosis is the most common cause of fatal community-acquired bacteremic pneumonia at the Royal Darwin Hospital in the Northern Territory of Australia.¹⁰²

Cases of human-to-human transmission are rare, but have been documented.^{103,104} The incubation period (time between exposure and appearance of clinical symptoms) is not clearly defined, but may range from 2 days to many years. Although serologic studies suggest that most infections with *B pseudomallei* are asymptomatic or mild,¹⁰⁵ individuals with risk factors, such as diabetes mellitus, alcoholism, cirrhosis, thalassanemia, or other immunosuppressed states, are at an increased risk of developing symptomatic infection. Other melioidosis-associated risk factors include chronic lung disease, excess kava consumption, and cystic fibrosis. Diabetes appears to be the most important of all the known risk factors because up to 50% of patients with melioidosis have diabetes mellitus.²⁴

Melioidosis, which presents as a febrile illness, has an unusually broad range of clinical presentations that has resulted in various classifications of melioidosis, none of which are considered satisfactory.¹⁰⁶ However, clinical disease with *B pseudomallei* is generally caused by bacteria spread and seeding to various organs within the host. The diversity of infectious presentations includes acute localized suppurative soft tissue infections, acute pulmonary infections, acute fulminant septicemia, and chronic localized infections.²⁴ 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.^{107,108}

Melioidosis is characterized by abscess formation. The majority of patients with melioidosis are septicemic. The lung is the most commonly involved organ—the nidus of infection is either a primary pneumonia or lung abscess, or the infection results from hematogenous seeding of the lung from bacteremia (Figure 7-2 and Figure 7-3). Patients with acute pulmonary melioidosis present with cough, fever, sputum production, and respiratory distress, and they 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



Fig. 7-2. Chest radiograph demonstrating a severe multilobar pneumonia.
Photograph: Courtesy of Bart Currie, MD, Royal Darwin Hospital, Australia.

shock may appear on presentation. In an Australian study of 252 prospective melioidosis cases in the Northern Territory of Australia, 46% of the cases presented with bacteremia; in these cases the mortality rate was 19%.¹⁰² Hematogenous seeding and abscess formation can occur in any organ (Figure 7-4). However, liver, spleen, skeletal muscle, prostate, and kidney are the most common abscess sites (Figures 7-5 and 7-6).²⁴

Less common presentations of melioidosis include uncomplicated infections of the skin (Figure 7-7), subcutaneous tissues, or the eye. Corneal ulcerations resulting from trauma, which become secondarily infected with *B pseudomallei*, are rapidly destructive.¹⁰⁹ Septic arthritis and osteomyelitis (Figure 7-8) have also been described, but cellulitis appears to be rare. In a prospective study of more than 2,000 patients in Thailand, primary meningitis or endocarditis was not observed, but meningitis secondary to cerebral abscess rupture and mycotic aneurysms was seen.²⁴ Other unusual melioidosis presentations include mediastinal masses, pericardial fluid collections, and adrenal abscesses.

The clinical presentation of melioidosis varies among different regions. In Thailand 30% of the melioidosis cases in children present as acute suppurative parotitis.¹¹⁰ These Thai 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.²⁴ Although acute suppurative parotitis is unusual

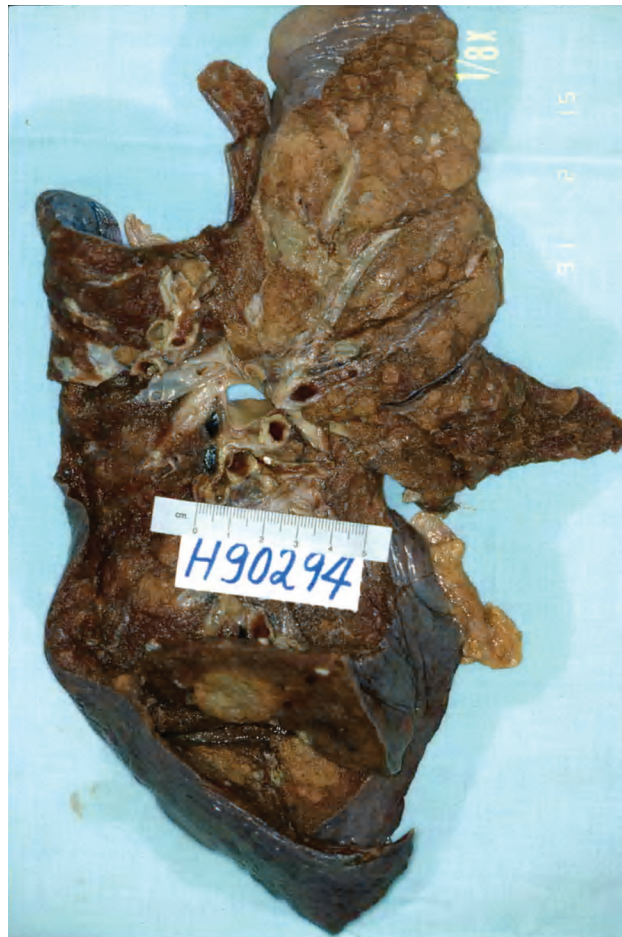


Fig. 7-3. Autopsy specimen demonstrating extensive pulmonary involvement with abscess formation resulting from *Burkholderia pseudomallei*.
Photograph: Courtesy of Bart Currie, MD, Royal Darwin Hospital, Australia.



Fig. 7-4. Pustules with an erythematous base resulting from septicemic melioidosis.
Photograph: Courtesy of Bart Currie, MD, Royal Darwin Hospital, Australia.

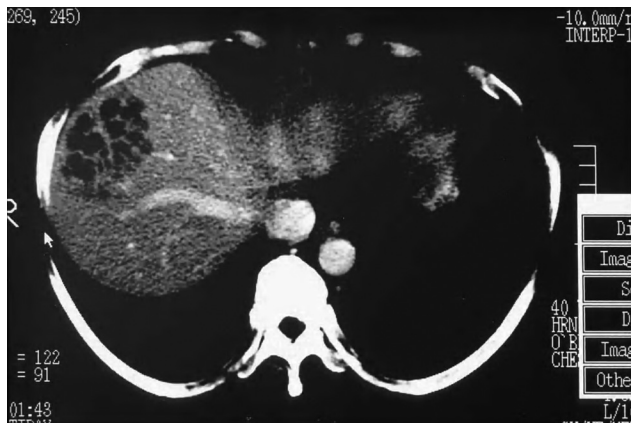


Fig. 7-5. Computed tomography scan showing multiloculated liver abscess.
 Photograph: Courtesy of Bart Currie, MD, Royal Darwin Hospital, Australia.

in Australia, approximately 4% of the melioidosis cases in northern Australia present as brain stem encephalitis with peripheral motor weakness or flaccid paraparesis. Features of this presentation include limb weakness, cerebellar signs, and cranial nerve palsies. Patients



Fig. 7-7. Skin lesions associated with melioidosis on the lower extremity.
 Photograph: Courtesy of Bart Currie, MD, Royal Darwin Hospital, Australia.

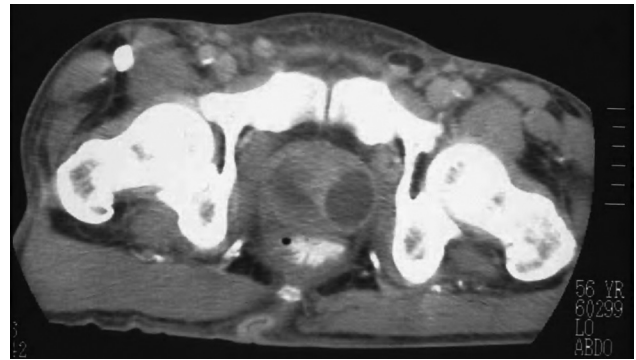


Fig. 7-6. Computed tomography scan showing prostatic abscess.
 Photograph: Courtesy of Bart Currie, MD, Royal Darwin Hospital, Australia.

with this syndrome usually have an initial normal state of consciousness. Multiple focal *B pseudomallei* microabscesses in the brain stem and spinal cord probably cause this syndrome.²⁴

Although acute infections in individuals with predisposing risk factors are the most common, latent infection with reactivation, resulting in an illness that



Fig. 7-8. Chronic osteomyelitis resulting from melioidosis.
 Photograph: Courtesy of Bart Currie, MD, Royal Darwin Hospital, Australia.

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.¹¹¹ 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.⁶⁴ 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 because he had not returned to an area of melioidosis endemicity after being imprisoned in northwest Thailand, nor been exposed to individuals with melioidosis.⁶³ A recent study of recurrent melioidosis cases in northeast Thailand demonstrated that 75% were caused by the same strain (relapse) and 25% resulted from reinfection with a new strain.¹¹² Infection with *B pseudomallei* does not protect susceptible individuals from reinfection with a new strain.

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.²⁰ The organism is usually detected in blood culture within 48 hours. Ashdown's medium, a crystal violet and gentamicin-containing medium that permits selective growth of *B pseudomallei* (see Figure 7-1), has been used to significantly increase the frequency of recovery of *B pseudomallei* from the rectum, wounds, and sputum as compared with recovery on blood and MacConkey agars.⁴⁷ 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.^{113,114} 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 lab. 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*,⁴⁶ although other investigators have reported mixed results.¹¹⁵ The Vitek 1 (bioMérieux) has also been found to be highly sensitive, having identified 99% of the 103 *B pseudomallei* isolates tested.¹¹⁶ However, in the same study, the Vitek 2 (bioMérieux) identified only 19% of these same isolates.¹¹⁶ *B pseudomallei* identification was more reliable using the Vitek 2 colorimetric GN card when the correct software was used to analyze the data.¹¹⁷

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 and false positives, because of a high antibody prevalence to *B pseudomallei* in healthy individuals, in endemic areas.¹⁰⁸ 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.¹¹⁸ Polymerase chain reaction-based identification techniques are also under development.^{119,120}

Treatment

Asymptomatic carriage probably does not occur except for the apparent residual respiratory colonization in some patients with cystic fibrosis.¹⁸ Therefore, the isolation of *B pseudomallei* from a clinical specimen requires treating the patient. All melioidosis cases—even mild disease—should be treated with initial intensive therapy (at least 2 weeks of intravenous [IV] therapy) followed by eradication therapy orally, for a minimum of 3 months. The choice of therapy for treating melioidosis is complicated because *B pseudomallei* is resistant to many antibiotics,^{121,122} including aminoglycosides, first- and second-generation cephalosporins, rifamycins, and nonureidopenicillins. *B pseudomallei* is also relatively insensitive to quinolones and macrolides.¹²³ Therapeutic options are therefore limited.

The first study demonstrating the effectiveness of ceftazidime for severe melioidosis was published in 1989. In this study,¹²⁴ ceftazidime treatment (120 mg/kg/d) 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/d), doxycycline (4 mg/kg/d), trimethoprim (10 mg/kg/d), and sulphamethoxazole (50 mg/kg/d) (TMP-SMX). In 1992 a second randomized study for severe melioidosis conducted

in Thailand also demonstrated a substantial reduction in mortality ($P = 0.04$) when ceftazidime plus TMP-SMX was used, as compared to the four-drug conventional therapy.¹⁰⁵

In 1999 a comparative treatment trial in Thailand found that imipenem was as effective as ceftazidime for treating 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.¹²⁵ Therefore, initial intensive therapy should be with high doses of ceftazidime (2 g IV every 6 hours, up to 8 g/d) or imipenem/cilastatin (1 g IV every 6 hours) or meropenem (1 g IV every 8 hours) combined with TMP-SMX (320 mg/1,600 mg IV or by mouth every 12 hours) for at least 14 days.¹⁰⁸ Critically ill patients with extensive pulmonary disease, organ abscesses, osteomyelitis, septic arthritis, or neurological melioidosis require longer intensive IV therapy.

The benefit of adding TMP-SMX to the initial antimicrobial regimen is supported by animal data and expert opinion.²³ However, a recent 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 TMP-SMX.¹²⁶ Nonetheless, all patients in the Northern Territory of Australia admitted to an intensive care unit for severe melioidosis are treated with meropenem and TMP-SMX. Meropenem is used rather than imipenem/cilastatin because it has fewer neurological side effects.¹²³

The median time to resolution of fever is 9 days, but patients with large abscesses or empyema often have fluctuating fevers longer than 1 month. 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.¹⁰² Adjunctive therapy with recombinant granulocyte colony-stimulation factor is routinely used for patients with melioidosis and septic shock in the Northern Territory of Australia. A retrospective review of mortality rates before and after the addition of granulocyte colony-stimulation factor therapy at the Royal Darwin Hospital was recently published. In this study, the introduction of granulocyte colony-stimulation factor as adjunctive therapy for patients with septic shock was associated with a decrease in mortality from 95% to 10%.¹²⁷ A randomized controlled trial to evaluate the efficacy of granulocyte colony-stimulation factor is under way in Thailand.¹⁰

After initial intensive therapy, oral maintenance therapy is given for another 12 to 20 weeks to prevent disease relapse. Oral maintenance therapy traditionally consists of chloramphenicol 40 mg/kg per day, doxycycline 4 mg/kg per day, and TMP-SMX 10 mg/50 mg/kg per day.¹²⁸ However, this combination frequently causes side effects resulting 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.¹⁰⁷ 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.¹²⁹ However, in the Northern Territory of Australia, TMP-SMX is used as monotherapy for maintenance therapy with a low relapse rate (1 failure in fewer than 60 patients).¹⁰² Trials underway in Thailand are comparing the efficacy of TMP-SMX monotherapy with combination therapy.

Quinolones are not recommended for first-line therapy for eradicating *B pseudomallei*. Ciprofloxacin and ofloxacin were found inferior, with a failure rate of 29% (95% confidence interval 17%–43%) when compared to a 20-week course of maintenance therapy consisting of amoxicillin/clavulanate or the combination of chloramphenicol, doxycycline, and TMP/SMX.¹³⁰ Another study also found that the combination of ciprofloxacin plus azithromycin was associated with an unacceptably high rate of relapse.¹³¹

Prevention

Several experimental melioidosis vaccines have been tested in rodent models of infection, including live attenuated vaccines, heterologous vaccines, acellular vaccines, and subunit vaccines.¹³² Variability in vaccination protocols, routes of challenge, and animal models makes 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.

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 *Bruceella melitensis*.^{133–137} *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.^{138,139} 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.^{138,140} 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*.¹⁴¹ In contrast, vaccination with purified *bipD* protein did not significantly protect this animal model.¹⁴¹ These studies suggest that live attenuated vaccines are promising candidates for melioidosis vaccines, but strains with defined deletion mutations should be examined 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 LD₅₀ of wild-type *B pseudomallei*.¹⁴² *B thailandensis* and *B pseudomallei* produce similar lipopolysaccharide (LPS) O-antigens and contain immunologically related secreted and cell-associated antigens,⁸⁹⁻⁹⁰ which probably account for the protection that *B thailandensis* affords. The *B pseudomallei* exopolysaccharide and capsular polysaccharide (see Exhibit 7-1) are not produced by *B thailandensis*, and both polysaccharides may be necessary for full protection against challenge with *B pseudomallei*. Live attenuated *F tularensis* strains were also tested as heterologous vaccine candidates against melioidosis in rodents.^{138,143} 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.¹⁴⁴ The vaccine consisted of a protein-polysac-

charide mixture (1:3), and it significantly protected hamsters against experimental challenge with virulent *B pseudomallei*. In addition, the acellular vaccine reduced melioidosis mortality in cetaceans from 45% to less than 1%.¹⁴⁴ Unfortunately, the exact chemical components of the vaccine were not well characterized, leaving a high probability of lot-to-lot variation.

In a recent study, mice were actively vaccinated with purified *B pseudomallei* capsular polysaccharide or LPS and challenged with virulent *B pseudomallei* by the intraperitoneal or aerosol route.¹⁴⁵ 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 capsule had an increased mean time to death, but 100% of the vaccinated mice were dead by day 28.¹⁴⁵ 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.^{81,100} Improved subunit vaccines that generate both humoral and cell-mediated immune responses are probably necessary to protect against infection with *B pseudomallei*.¹⁴⁶

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.¹⁸ Avoidance of *B pseudomallei* in the environment by those individuals with known risk factors is the only proven method of disease prevention. Animal studies have demonstrated the protective efficacy of doxycycline and to a lesser extent, ciprofloxacin, as prophylaxis against experimental melioidosis.¹⁴⁷ Based on these animal data, either doxycycline 100 mg by mouth twice daily or ciprofloxacin 500 mg by mouth twice daily may be recommended to individuals with risk factors and exposure to *B pseudomallei*. However, no clinical evidence suggests the efficacy of antibiotic prophylaxis in the prevention of human melioidosis.

SUMMARY

A disease caused by the gram-negative bacterium *B pseudomallei*, melioidosis 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 it is spread to humans through direct contact with the contaminated source and/or through inhalation. The majority of melioidosis cases have an identifiable risk factor, such as diabetes mellitus, alcoholism, cirrhosis, or other immunosuppressed states, although healthy people may develop disease. The incubation period is

not clearly defined, but may range from 2 days to many years. Exposed individuals with a subclinical infection are potentially at risk for reactivation.

Melioidosis has an unusually broad range of clinical presentations. Disease is generally caused by bacteria spread and seeding to various organs within the host. Melioidosis is characterized by abscess formation. The majority of patients with melioidosis are septicemic. 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 isolate *B pseudomallei* from 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 antibiot-

ics. Antibiotics recommended to treat melioidosis are ceftazidime, imipenem/cilastatin, or meropenem, each in combination with 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 postexposure prophylaxis in preventing human disease after exposure is unknown.

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Chapter 8

TULAREMIA

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INTRODUCTION

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USE OF TULAREMIA AS A BIOLOGICAL WEAPON

SUMMARY

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INTRODUCTION

Francisella tularensis poses a substantial threat as a biological weapon, and it is viewed by most experts as a dangerous pathogen if weaponized. Both the United States and the former Soviet Union developed weaponized *F tularensis* during the Cold War.^{1,2} It is unclear whether tularemia has ever been used deliberately as a biological weapon. The Japanese experimented with *F tularensis* as a biological weapon, but there is no documentation of its use in military operations.³ There is also speculation that the former Soviet Union used *F tularensis* as a weapon against German troops in the Battle of Stalingrad during World War II.² Despite the tularemia outbreak among soldiers of both armies during this battle, some authors suggest that natural causes, as opposed to an intentional release, were responsible for the epidemic.⁴ There was

also speculation that *F tularensis* was used as a biological weapon by Serbia in the Kosovo conflict, although the subsequent investigation suggested the observed cases were not caused by an intentional release.^{5,6}

F tularensis has been included in the list of Centers for Disease Control and Prevention Category A threat organisms because of the infectivity with exposure to low numbers of organisms, the ease of administration, and the serious consequences of infection.¹ Tularemia's effectiveness as a biological weapon includes a nonspecific disease presentation, high morbidity, significant mortality if untreated, and the limited ability to obtain a rapid diagnosis. Although tularemia responds to antibiotics, the use of an antibiotic-resistant strain can make these countermeasures ineffective.

INFECTIOUS AGENT

Tularemia was named after 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 tularensis*.⁷ Wherry and Lamb subsequently identified the pathogen as the cause of conjunctival ulcers in a 22-year-old man.⁸ Edward Francis made significant scientific contributions to the understanding of the disease in the early 20th century, including naming it "tularemia."⁹

F tularensis is an aerobic, gram-negative coccobacilli. *F tularensis* is not motile, and appears as small (approximately 0.2–0.5 μm by 0.7–1.0 μm),¹⁰ faintly staining gram-negative bacteria on Gram's stain (Figure 8-1). *F tularensis* was formerly included in the *Pasteurella* and the *Brucella* genera. Eventually a new genus was created, and the name *Francisella* was proposed in tribute to Edward Francis.¹¹ A closely related species, *Francisella philomiragia*, has also been described as a human pathogen.^{12,13} *F tularensis* is considered to have four subspecies: (1) *tularensis*, (2) *holarctica*, (3) *mediasiatica*, and (4) *novicida*.¹⁴ *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. Subpopulation A.I. causes disease in the central United States, and subpopulation A.II. is found mostly in the western United States.¹⁵ *F tularensis* subspecies *holarctica* (formerly described as *palaearctica*), also known as Type B (or biovar B), is found in Europe and Asia, but also occurs in North America. *F tularensis* subspecies *holarctica* causes a less virulent form of disease than subspecies *tularensis*, but has been documented to

cause bacteremia in immunocompetent individuals.^{16,17} Before antibiotics, *F tularensis* subspecies *tularensis* resulted in 5% to 57% mortality, yet *F tularensis* subspecies *holarctica* was rarely fatal.¹⁸ Unlike these other subspecies, *F novicida* rarely causes human disease.¹² *F tularensis* subspecies *mediasiatica* has 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*.^{19,20} The four subspecies can be distinguished with biochemical tests and genetic analysis.

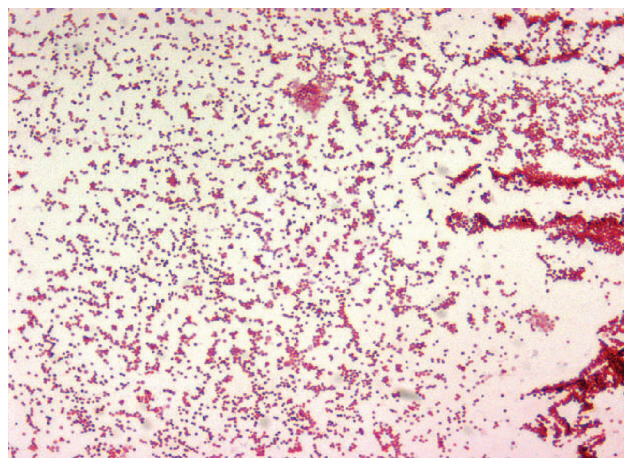


Fig. 8-1. Gram's stain of *Francisella tularensis*. Photograph: Courtesy of Dr Larry Stauffer, Oregon State Public Health Laboratories, Centers for Disease Control and Prevention, Atlanta, Georgia, Public Health Image Library, #1904.

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 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 tularemia 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. The investigation should yield the likely cause of the outbreak, which could be varied (exposure to infected animals, arthropod-borne, etc). By determining the cause of tularemia, 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 common *F tularensis* subspecies causing clinical tularemia in North America.¹⁰ Type A was once thought not to occur in Europe, but a type A strain has recently been isolated from flea and mite parasites of small rodents trapped in Slovakia.²² *F tularensis* subspecies *holarctica* (type B), found throughout the Northern Hemisphere, is less pathogenic.¹ In the United States an average of 124 tularemia cases per year were reported from 1990 through 2000.²³ Over half of all cases reported came from Arkansas, Missouri, South Dakota, and Oklahoma, where the foci of infection are well-established. Tularemia can be transmitted 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.

Various epidemiological categories of tularemia have been suggested, often dependent on the infective vector, mode of infection, or occupation of the infected individuals.¹⁸

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.²⁴

Food and Water Ingestion

Tularemia can also be contracted by eating meat from infected animals⁹ or food contaminated by infected animals.²⁵ Water can also become contaminated from animals infected with tularemia and cause human infection. During March through April 1982, 49 cases of oropharyngeal tularemia were identified in Sansepolcro, Italy.²⁶ 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.²⁶ Waterborne transmission of ulceroglandular tularemia also occurred during a Spanish outbreak among 19 persons who had contact with river-caught crayfish.²⁷ Contaminated water may have contributed to recent outbreaks of oropharyngeal tularemia in Turkey²⁸ and Bulgaria.²⁵ 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*.²⁹

Mammalian Bites and Arthropod Vectors

Mammalian bites are another source of tularemia transmission to humans. Instances of human transmission from the bites or scratch of a cat, coyote, ground squirrel, and a hog were documented over 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.³⁰

Transmission of tularemia by the bites of ticks and flies is also well-documented.¹⁰ *Dermacentor* species

ticks (dog ticks) are important vectors in areas where enzootic transmission occurs in North America³¹ and Europe.³² *Ixodes* species ticks may also contribute to *F tularensis* transmission.³³ In Utah during the summer of 1971, 28 of 39 tularemia cases were contracted from deerfly (*Chrysops discalis*) bites.³⁴ 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*).³⁵

Aerosol Transmission

The largest recorded pneumonic tularemia outbreak occurred in Sweden during the winter of 1966 through 1967, when 676 cases were reported.³⁶ 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.³⁷ 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.³⁸ 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.³⁸ The strong epidemiological link with grass cutting adds plausibility to this explanation.³⁹ 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.³⁸

The only other previously reported outbreak of pneumonic tularemia in the United States occurred at Martha's Vineyard during the summer of 1978.⁴⁰ 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,⁴⁰ and pneumonic tularemia was initially reported in Massachusetts in 1947.⁴¹

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.⁴² 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 over 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.⁴³ 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 over 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.^{5,6} The Kosovo outbreak and subsequent investigation are described in detail in chapter 3, 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 1921.⁴⁴ Tularemia is the third most commonly acquired laboratory infection,⁴⁵ and recent laboratory-acquired infections of tularemia emphasize the laboratory hazard that this organism presents.⁴⁶ 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

For infection to occur, bacterial pathogens must traverse the normal skin and mucosal barriers that typically prevent microorganisms from entering the body. Breaks in the skin from lacerations or abrasions provide opportunity for *F tularensis* transmission and infection. Arthropod vectors can bypass the skin defenses with

a bite, thus inoculating the organism directly into the host. However, the portal of entry can also be mucous membranes in the respiratory tract, ocular membranes, or the gastrointestinal tract.

One of the remarkable attributes of *F tularensis* is the low infectious dose. As few as 10 organisms can produce an infection when injected subcutaneously into human volunteers, and only 10 to 50 organisms are required when administered to human volunteers by aerosol.^{47,48} Recent investigations have attempted to elucidate the unique characteristics that allow *F tularensis* to cause infection at such a low number of organisms. As an intracellular pathogen, *F tularensis* has developed the means to survive in the typically hostile environment inside macrophages by interfering with multiple aspects of macrophage function. On initial entry into the macrophage, *F tularensis* uses a bacterial acid phosphatase, AcpA, to inhibit the bactericidal respiratory burst response of the macrophage.^{49,50} Additionally, both *F tularensis* Type A and B can inhibit acidification of the phagosome after entry into the macrophage, escape from the phagosome, and reside in the macrophage cytoplasm.^{51,52} Another survival mechanism of *F tularensis* is the interference with the normal macrophage response by inhibiting Toll-like receptor signaling and cytokine secretion, as demonstrated in experiments with murine macrophages and the live vaccine strain (referred to as LVS, which is subspecies *holarctica* or a Type B strain) of *F tularensis*.⁵³ An absence of Toll-like receptor signaling inhibits the typical robust innate immune response that could eliminate the bacteria. Replication of the organism in the macrophage begins slowly, but eventually large numbers of organisms can be found in a single macrophage.^{52,54,55} 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.⁵⁶ Researchers have identified only some of the factors required by *F tularensis* for survival in macrophages, including *IglC*, a 23-kDa protein that most likely affects Toll-like receptor-4 signal transduction,^{53,57} and the *MglAB* operon that regulates transcription of virulence factors.⁵⁸ The MinD protein functions as a pump for substances containing free radicals such as hydrogen peroxide, allowing the organism to resist oxidative killing.⁵⁹

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 LVS⁶⁰ and tumor necrosis factor- α is at least as impor-

tant as interferon- γ for control of *F tularensis* infection.⁶¹ The host defense within macrophages appears to be crucial at controlling infection by *F tularensis*. In human monocytes/macrophages, LVS strain and *F novicida* induced the processing and release of interleukin (IL)-1 β , an essential component of the inflammatory immune response.⁶³ However, killed bacteria did not induce this response, but did induce the early phases required for IL-1 β , such as mRNA transcription. The results suggest that only live *Francisella* can escape from the phagosome, and thus trigger the function of caspase-1, which converts the precursor of IL-1 β to its active form. In mice deficient in caspase-1 as well as ASC, an adaptor protein involved in host cell death, substantially higher bacterial loads were observed, as well as early mortality, compared to normal mice.⁶⁴ Neutrophils perform an important function in limiting the spread of *F tularensis* after inoculation. Experiments have demonstrated that neutrophils can kill *F tularensis*,⁶⁵ and mice depleted of neutrophils appear susceptible to infection with *F tularensis* LVS.⁶⁶

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.⁶⁷ 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 are required.⁶⁸ Vaccination with *F tularensis* LVS appears to produce a long-term memory T-cell response (as measured by lymphocyte stimulation),⁶⁹ 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*.⁶⁰ The protective memory response is dependent on a robust proinflammatory cellular response, because administration of anti-interferon- γ and anti-tumor necrosis factor- α antibodies to previously vaccinated mice dramatically lowers the lethal infective intradermal dose of *F tularensis*.⁶² This response initially appears 2 to 4 weeks after initial infection,^{70,72} and it can remain detectable for many years.^{69,73}

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.⁷⁴ When laboratory workers received a formalin-killed whole-cell vaccine developed by Foshay et al.,⁷⁵ a strong humoral response was elicited but was not protective against cutaneous⁴⁸ or respiratory⁴⁷ 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.⁷⁰ Investigations focused on identifying protective antigens are ongoing, particularly in animal models.¹⁴ 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.⁷⁴ 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.

In addition to understanding the interaction of *F tularensis* with the immune system, substantial research has focused on the poorly understood virulence factors of *F tularensis*.¹⁰ The lipopolysaccharide capsule of many gram-negative pathogens elicits a profound proinflammatory immune response, which can lead to the clinical manifestations of septic shock.⁷⁶ 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,⁷⁷ in contrast to lipopolysaccharide from other gram-negative pathogens. *F tularensis* does have virulence factors allowing for survival within macrophages and possibly other cells, and *iglC*, *mglAB*, and *minD* genes were previously mentioned. Advances in genetic manipulation will enhance understanding of the role of specific genes in the pathogenesis of this organism.¹⁴ One promising technique is allelic replacements, with successful studies on *F tularensis* recently conducted.⁷⁸

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.⁷⁹ The disease manifestations of tularemia have been classified into two groups (ulceroglandular and typhoidal),⁸⁰ or more specific categories (ulceroglandular, glandular, oculoglandular, oropharyngeal, typhoidal, pneumonic, and septic). The more specific classification is preferred^{1,11} because direct pulmonary inoculation is probably a presentation clinically distinct from the nonspecific syndrome of typhoidal tularemia. Symptoms overlap among these seven categories.⁸¹ Patients with glandular tularemia forms (ulceroglandular and oculoglandular) usually present with ulcerative skin lesions. However, a dis-

tinct clinical presentation of lymphadenopathy greater than 1 cm and no skin lesions is well-described and known as glandular tularemia. Patients with typhoidal tularemia lack mucosal or cutaneous lesions and are less likely to present with lymphadenopathy, but have various systemic symptoms including fever, weight loss, and possible signs of an atypical pneumonia.¹⁰

Clinical symptoms in cases of ulceroglandular tularemia typically appear after an incubation period of 3 to 6 days.⁸⁰ These manifestations of disease include fever (85% of cases), chills (52% of cases), headache (45% of cases), cough (38% of cases), and myalgias (31% of cases). The fever may be associated with pulse-temperature disassociation (42% of cases in one series)⁸⁰ (the pulse increases fewer than 10 beats per minute per 1 $^{\circ}$ F increase in temperature above normal), although 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.^{80,81}

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.⁸⁰ Lesions are typically associated with regional lymphadenopathy, and a lack of lymphadenopathy may suggest another etiologic agent.⁸⁰ 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.⁸¹

Oculoglandular tularemia is similar to the ulceroglandular form, 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.⁸² Food and water contamination can also lead to oculoglandular infection.²⁵

In one series pharyngitis was observed in 24% of patients with tularemia.⁸⁰ Possible findings on examination include erythema, exudates, petechiae, hemorrhage, or ulceration. Other findings may include retropharyngeal abscess or suppuration of the regional lymph nodes. The nonspecific mild symptoms of pharyngitis associated with the other forms of tularemia should be distinguished from the severe, usually exudative, pharyngitis of the oropharyngeal form of tularemia.⁸¹ 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)²⁸ and Bulgaria (89% of cases),²⁵ 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%.^{80,83} 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 or glandular tularemia, with an onset ranging from a few days to months after the appearance of initial nonpulmonary symptoms.⁸³ Approximately 30% of patients with ulceroglandular disease and 80% of patients with typhoidal tularemia also have pulmonary signs and/or symptoms consistent with pneumonia.⁸⁰ Pneumonic tularemia can also occur from direct inhalation of the organism, which has been demonstrated in human experimental models.^{47,84} 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.⁸⁴ Chest radiographic findings in pneumonic tularemia are highly variable and nonspecific⁸⁵ because they can mimic findings in bacterial pneumonias, tuberculosis, lymphoma, or lung carcinoma.⁸³ 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, cavitory lesions, and bronchopleural fistulas.

A recent 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 over a month later while searching for the cause of another pneumonic summer illness.^{37,86} 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.⁸⁷ A decade earlier, of the 96 patients with tularemia presenting to a Veteran's Hospital in Arkansas, five had pneumonic tularemia.⁸⁸

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.⁸¹ 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.^{79,80,89}

The laboratory findings with tularemia are nonspecific. Hemoglobin and platelet counts are typically normal, and the white blood cell count is usually only mildly elevated, with no alteration in the normal cell differential.⁸⁰ Microscopic pyuria may be observed.⁸⁰ One case series describes tularemia associated with skeletal muscle abscesses, elevated creatine kinase, and rhabdomyolysis.⁹⁰ 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.⁸⁰

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%.⁸¹ 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 the clinical microbiology laboratory because of the high incidence of laboratory-acquired infection⁹¹ (see "Issues for Laboratory Workers").

Diagnosis

The diagnosis of tularemia is difficult because the clinical presentations for the various forms are not specific and diagnostic modalities 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 judgment⁹² or epidemiological syndromic surveillance⁹³ 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,⁹⁴⁻⁹⁶ cultures from ulcer sites, sputum, gastric washings, and pharyngeal and conjunctival exudates are usually negative.¹⁰ *F tularensis* is difficult to grow using standard media, but medium supplemented with cysteine or other sources of sulfhydryl groups can enhance recovery.^{10,97} Cysteine glucose blood agar has been the traditional medium of choice.⁹⁸ Charcoal yeast extract agar and Thayer-Martin agar are two additional preparations that may support the growth of *F tularensis*. The colonies appear gray-white on chocolate or Thayer-Martin agar (Figure 8-2). The organism is optimally grown in a CO₂ incubator and tends to grow more slowly than bacteria routinely encountered in clinical practice. 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* do not have these fastidious growth requirements.⁹⁹ The organism may be identified with biochemi-



Fig. 8-2. 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, #1912.

cal testing, but automated identification systems in microbiology laboratories may misidentify the pathogen.¹⁰⁰ The samples should be referred to a specialized laboratory. Blood cultures are rarely positive, even in cases of severe disease.¹⁶ Occasionally, positive blood cultures have been observed in immunocompromised persons (infected with the less virulent subspecies *holarctica*), and have been discovered when blind subculture of blood cultures has been conducted.⁹⁶

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.⁸⁰

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 non-specific presentation.¹⁰¹ 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^{102,103} and the genus-specific *tul4* gene encoding a 17-kd membrane lipoprotein.^{102,104-106} Other PCR assays have been designed to target *fopA*, a locus encoding an outer membrane protein.^{104,107,108} PCR testing of tissue specimens has been performed with mouse models,¹⁰⁹ rabbit tissue,¹¹⁰ and humans with ulceroglandular tularemia.^{102,111} 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 inhibitors¹⁰ or other unknown confounding factors. Antigen-detection techniques have also been suggested for *F tularensis*,^{110,112} 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.

Treatment

Antibiotics usually provide curative therapy for tularemia, with resulting mortality rates of only 1% to 2.5%.^{1,80} 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.^{1,10,113} Relapses with streptomycin rarely occur. Gentamicin or other aminoglycosides are thought to be as effective as streptomycin and are often listed as reasonable alternatives in clinical practice reviews,^{1,114,115} but no controlled trials have been reported. Beta-lactam antibiotics such as ceftriaxone¹¹⁴ 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.^{1,101,114} Chloramphenicol is another alternative,¹ but it is rarely used in the United States. The fluoroquinolones offer an additional treatment option,¹¹⁶⁻¹¹⁸ especially with the high bioavailability of oral preparations. Although extensive clinical data are lacking for the fluoroquinolones, one report of a tularemia outbreak in Spain noted a 5% failure rate for ciprofloxacin, compared to a 23% failure rate for streptomycin and 43% failure rate for doxycycline.¹⁰¹ However, the number of patients treated with streptomycin in this study was 94, compared to only 22 being treated with ciprofloxacin. 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 8-1. 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

TABLE 8-1
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.

Source: Dennis DT, Inglesby TV, Henderson DA, et al. Tularemia as a biological weapon: medical and public health management. *JAMA*. 2001;285:2763-2773.

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.¹¹⁹ 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 8-2.

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.¹²⁰ This vaccine, developed from a Type B strain, was transferred in 1956 to the United States,¹²¹ where researchers Eigelsbach and Downs further characterized the strain, designating it as the LVS of *F tularensis*.¹²² 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 hundreds 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-dose respiratory challenge and partially protected against high-dose respiratory challenge with virulent

Type A strains.^{47,84} 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.⁶⁰ 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.¹²³ Injections of bacterial DNA (as unmethylated CpG motifs) can also confer a similar early protective response.¹²⁴ The prompt development of immunity after vaccination in mice suggests that the protective mechanisms are attributable to innate immunity⁶⁰ because an adaptive response would require 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.

TABLE 8-2
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 Bio-defense.

[†]Usage is not approved by the Food and Drug Administration. Source: Dennis DT, Inglesby TV, Henderson DA, et al. Tularemia as a biological weapon: medical and public health management. *JAMA*. 2001;285:2763-2773.

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.⁷⁵ 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.⁷⁴

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 has some efficacy, as documented in a human challenge

model; however, this protection is not 100%, particularly at high-dose aerosol challenges.^{47,84} In addition, an epidemiological study showed that the incidence of typhoidal tularemia in laboratory workers decreased after the introduction of vaccination with LVS.¹²⁵ The primary disadvantages are the potential hazards associated with a live vaccine (such as potential dissemination and 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.^{10,14} Promising vaccine candidates are being explored.¹⁴

ISSUES FOR LABORATORY WORKERS

Tularemia is considered a significant hazard for laboratory workers.⁹¹ All experiments that involve using the live virulent form of *F tularensis* should be conducted in biosafety level 3 containment. Additionally, vaccination may augment personal protective measures in diminishing the risk of laboratory-acquired infections. A retrospective review of tularemia cases at USAMRIID was conducted, documenting that typhoidal tularemia incidence dropped substantially after the live vaccine was instituted,

decreasing from 5.70 to 0.27 cases per 1,000 at-risk employee-years.¹²⁵ 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.¹²⁵ 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.¹²⁶

USE OF TULAREMIA AS A BIOLOGICAL WEAPON

Tularemia could be used as a biological weapon in a number of 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, were 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.¹²⁷ 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 tularemia 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 Tularemia may also be confused with another biological weapon. Epidemiological clues to distinguish tularemia from plague or anthrax is the clinical course (slower with tularemia), case fatality rate (higher with plague¹²⁸ or anthrax¹²⁹), and possibly the pattern of pulmonary manifestations observed on chest radiograph, such as the large pleural effusions and mediastinal widening characteristic of inhalational anthrax.¹³⁰ Pulmonary tularemia may be difficult to distinguish from Q fever, another potential biological weapon agent.

SUMMARY

Tularemia constitutes a substantial threat as a biological weapon. The variety of clinical manifestations of tularemia 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 coun-

termeasures allow healthcare professionals to minimize the impact of its use. Although the current LVS vaccine provides a preventive option against tularemia, 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 9

BRUCELLOSIS

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INTRODUCTION

Brucellosis is a zoonotic infection of domesticated and wild animals caused by organisms of the genus *Brucella*. Humans become infected by ingesting animal food products, directly contacting infected animals, or inhaling infectious aerosols either by accident or as a result of bioterrorism.

Military medicine has played a major role in studying and describing brucellosis in humans.¹ 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.² Three additional British army surgeons working on the island of Malta during the 1800s were responsible for important observations of the disease. JA Marston described clinical characteristics of his own infection in 1861.³ In 1887 David Bruce, for whom the genus *Brucella* is named, isolated the causative organism from the spleens of five patients who died from the disease and placed the microorganism within the genus *Micrococcus*.⁴ Ten years later, ML Hughes, who coined the name "undulant fever," published a monograph that detailed clinical and pathological findings in 844 patients.⁵

That same year, Danish investigator B Bang identified an organism, which he called the "bacillus of abortion," in the placentas and fetuses of cattle suffering from contagious abortion.⁶ In 1917 AC Evans recognized that Bang's organism was identical to that described by Bruce as the causative agent of human brucellosis. The organism infects mainly cattle, sheep, goats, and other ruminants, in which it causes abortion, fetal death, and genital infections.^{7,8} 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. Because of the

worldwide distribution of brucellosis, international travel and military deployments increase the risk of exposure.⁹ The disease frequently becomes chronic and may relapse, even with treatment. Laboratory-acquired infections have been documented as awareness of this disease has increased.¹⁰⁻¹³ Laboratory accidents may become more frequent and significant as biodefense research expands in the academic and biotechnology industries. Strict adherence to proper engineering controls, good laboratory and microbiology techniques, and personal protective equipment, in addition to vaccination (when possible), significantly reduce the incidence of laboratory-acquired infections.^{14,15} However, no human brucellosis vaccine is available for laboratory workers.

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 in 1944 and 1945 with animal targets. By 1969 the United States terminated its offensive *Brucella* program and destroyed all its biological weapon munitions. Although the munitions developed were never used in combat, studies conducted under the offensive program reinforced the concern that *Brucella* organisms might be used against US troops as a biological warfare agent.¹⁶ Even before the 2001 anthrax attacks, civilian populations were recognized as potential high-yield targets. A 1997 model of aerosol attack with *Brucella* on an urban population included an estimated economic impact of \$477.7 million per 100,000 persons exposed.¹⁷ *Brucella* represents one of many biological agents of zoonotic disease that could pose a threat as a terrorist weapon against human or agricultural targets.¹⁸ An excellent review of brucellosis was published in 2005.¹⁹

INFECTIOUS AGENT

Brucellae are small, nonmotile, nonsporulating, nontoxigenic, nonfermenting, facultative, intracellular, gram-negative coccobacilli parasites that may, based on DNA homology, represent a single species.^{20,21} Taxonomically, brucellae are classified as α -*Proteobacteria* and subdivided into six species, each comprising several biovars.²² Each species has a characteristic, but not absolute, predilection to infect certain animal species (Table 9-1). *Brucella melitensis*, *B suis*, *B abortus*, and *B canis* are the classic causative agents of disease in humans. Human infection with recently discovered

marine strains (see Table 9-1) has also been noted.²³

Human infections with *Brucella ovis* and *Brucella neotomae* 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,²⁴ 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

TABLE 9-1
TYPICAL HOST SPECIFICITY OF *BRUCELLA* SPECIES

<i>Brucella</i> Species	Animal Host	Human Pathogenicity
<i>B suis</i>	Swine	High
<i>B melitensis</i>	Sheep, goats	High
<i>B abortus</i>	Cattle, bison	Intermediate
<i>B canis</i>	Dogs	Intermediate
Marine species	Marine mammals	Rare
<i>B ovis</i>	Sheep	None
<i>B neotomae</i>	Rodents	None

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; production of hydrogen sulfide; growth in the presence of thionine or basic fuchsin dyes; agglutination by antisera directed against certain lipopolysaccharide (LPS) epitopes; and susceptibility to lysis by bacteriophage. *Brucella* can grow on blood agar plates and does not require X or V factors for growth. Analysis of fragment lengths of DNA cut by various restriction enzymes has also been used to differentiate brucellae groupings.²¹ Recent studies using proteomics, complete genomic sequencing, and multilocus analysis

of variable number tandem repeats have rapidly expanded information on virulence determinants, identification of pathogenicity islands, and evolutionary relatedness among the *Brucella*.²⁵⁻³⁰

The LPS component of the outer cell membranes of brucellae is different—both structurally and functionally—from that of other gram-negative organisms.^{31,32} The lipid A portion of a *Brucella* organism LPS contains fatty acids that are 16-carbons long, and it lacks the 14-carbon myristic acid typical of lipid A of *Enterobacteriaceae*. This unique structural feature may underlie the remarkably reduced pyrogenicity of *Brucella* LPS, compared with the pyrogenicity of *Escherichia coli* LPS (less than 1/100th).³³ In addition, the O-polysaccharide portion of LPS from smooth organisms contains an unusual sugar, 4,6-dideoxy-4-formamido- α -D-mannopyranoside, which is expressed either as a homopolymer of α -1,2-linked sugars (A type), or as a repetitive series of 3- α -1,2 and 2- α -1,3-linked sugars (M type). These variations in O-polysaccharide linkages lead to specific, taxonomically useful differences in immunoreactivity between A and M sugar types.³⁴ A unique feature of this organism, unlike most pathogenic bacteria, is the lack of many classical virulence factors, such as exotoxins; capsule; flagella; fimbriae; plasmids; lysogenic phage; antigenic variation; cytolysins; pathogenic islands; or type I, II, or III secretion systems; making characterization of pathogenic mechanisms in this organism highly challenging. Recently, however, a type IV secretion system³⁵ has been identified as an important contributor to virulence.

DISEASE

Epidemiology

Animals may transmit *Brucella* organisms during septic abortion, during slaughter, and through their milk. Brucellosis is rarely, if ever, transmitted from person to person. The incidence of human disease is thus closely tied to the prevalence of infection in sheep, goats, and cattle, and to practices that allow exposure of humans to potentially infected animals or their products. In the United States, where most states are free of infected animals and where dairy products are routinely pasteurized, illness occurs primarily in individuals who have occupational exposure to infected animals, such as veterinarians, shepherds, cattlemen, and slaughterhouse workers. 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 Kuwait, for example, disease with a relatively high proportion of respiratory complaints has occurred in individuals who have camped in the desert during the spring lambing season.³⁶ In Australia an outbreak of *B suis* infection was noted in hunters of infected feral pigs.³⁷ *B canis*, a naturally rough strain that typically causes genital infection in dogs, can rarely infect humans.³⁸

Brucellae are highly infectious in laboratory settings; numerous laboratory workers who culture the organism have become infected. However, fewer than 200 total cases per year (0.04 cases per 100,000 population) are reported in the United States. The incidence is much higher in other regions such as the Middle East; countries bordering the Mediterranean Sea; and China, India, Mexico, and Peru. Jordan, for example, had 33 cases per 100,000 persons in 1987; Kuwait had 88 cases per 100,000 persons in 1985; and Iran had 469 cases from 1997 to 2002.³⁹⁻⁴¹

Pathogenesis

Brucellae can enter mammalian hosts through skin abrasions or cuts, the conjunctiva, the respiratory tract, and the gastrointestinal tract.⁴² In the gastrointestinal tract, the organisms are phagocytosed by lymphoepithelial cells of gut-associated lymphoid tissue, from which they gain access to the submucosa.⁴³ Organisms are rapidly ingested by polymorphonuclear leukocytes, which generally fail to kill them,^{44,45} and are also phagocytosed by macrophages (Figure 9-1). Bacteria transported in macrophages, which travel to lymphoid tissue draining the infection site, may eventually localize in lymph nodes, liver, spleen, mammary glands, joints, kidneys, and bone marrow.

In macrophages, brucellae inhibit fusion of phagosomes and lysosomes,⁴⁶ and replicate within compartments that contain components of endoplasmic reticulum⁴⁷ via a process facilitated by the type IV secretion system.³⁵ If unchecked by macrophage microbicidal mechanisms, the bacteria destroy their host cells and infect additional cells. Brucellae can also replicate extracellularly in host tissues. Histopathologically, the host cellular response may range from abscess formation to lymphocytic infiltration to granuloma formation with caseous necrosis.

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

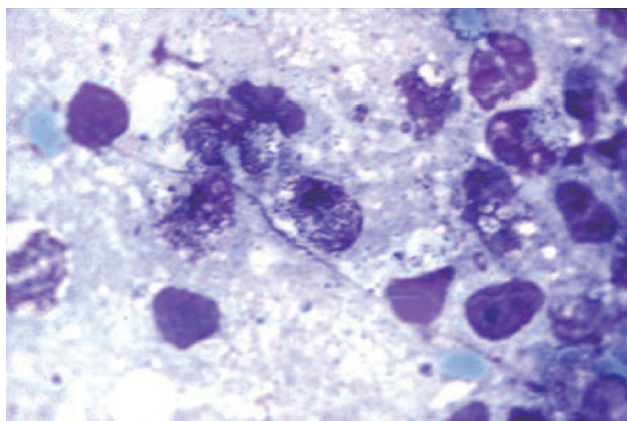


Fig. 9-1. Impression tissue smear from a bovine aborted fetus infected with *Brucella abortus*. The bacteria appear as lightly stained, gram-negative cells.

Photograph: Courtesy of John Ezzell, PhD, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

with a long O-polysaccharide side chain); *B melitensis* may be less susceptible than *B abortus* to complement-mediated killing.^{48,49} Administration of antibody to mice before challenge with rough or smooth strains of brucellae reduces the number of organisms that appear in the liver and spleen. This effect is attributable mainly to antibodies directed against LPS, with little or no contribution of antibody directed against other cellular components.⁵⁰

Reduction in intensity of infection in mice can be transferred from immune to nonimmune animals by both cluster of differentiation 4⁺ (CD4⁺) and CD8⁺ T cells⁵¹ or by the immunoglobulin (IgG) 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.^{52,53} Neutralization of *B abortus*-induced host interferon gamma (IFN- γ) during infection in pregnant mice prevents abortion.⁵⁴ Moreover, macrophages treated with IFN- γ in vitro inhibit intracellular bacterial replication.⁵⁵ Studies in humans support a role for IFN- γ in protection; homozygosity for the IFN- γ + 874A allele is associated with about a 2-fold increase in the incidence of brucellosis.⁵⁶ In ruminants, vaccination with killed bacteria provides some protection against challenge, but live vaccines are more effective.⁵⁷⁻⁵⁹ The most efficacious live vaccines express surface O-polysaccharide; at a minimum, a complete LPS core is required for rough mutant vaccine efficacy against *B abortus* and *B ovis* infections in the mouse model.⁶⁰

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.⁶¹ 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 in the placenta of erythritol may further enhance growth of brucellae. Products of conception at the time of abortion may contain up to 10¹⁰ bacteria per gram of tissue.⁶² When septic abortion occurs, the intense concentration of bacteria and aerosolization of infected body fluids during parturition often result in infection of other animals and humans.

Clinical Manifestations

Clinical manifestations of brucellosis are diverse, and the course of the disease is variable.⁶³ Patients with brucellosis may present with an acute, systemic febrile illness; an insidious chronic infection; or a localized inflammatory process. 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 9-2). 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 occur. 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 be found. Brucellosis does not usually cause leukocytosis. Some patients may be moderately neutropenic⁶⁴; however, cases of pancytopenia have been noted.⁶⁵ In addition, bone marrow hypoplasia, immune thrombocytopenic purpura, and erythema nodosum may occur during brucellosis infections.⁶⁶⁻⁶⁸ 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% of patients, particularly in young adults.⁶⁹⁻⁷¹ 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.⁷² 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 rarely occurs. In contrast with frequent infection of the axial skeleton, osteomyelitis of long bones is rare.⁷³

Infection of the genitourinary tract (an important target in ruminant animals) may lead to pyelonephritis, cystitis, Bartholin's gland abscess and, in males, epididymo-orchitis. Both pyelonephritis and cystitis may mimic their tuberculous counterparts, with "sterile" pyuria on routine bacteriologic culture.⁷⁴⁻⁷⁶ With bladder and kidney infection, *Brucella* organisms can be cultured from the urine. Brucellosis in pregnancy can lead to placental and fetal infection.⁷⁷ 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, including mostly cough, dyspnea, or pleuritic pain, chest radiograph examinations are usually normal.⁷⁸ Diffuse or focal infiltrates, pleural effusion, abscess, and granulomas may be seen.

Hepatitis and, rarely, liver abscess also occur. Mild elevations of serum lactate dehydrogenase and alkaline phosphatase are common. Serum transaminases are frequently elevated.⁷⁹ Biopsy may show well-formed granulomas or nonspecific hepatitis with collections of mononuclear cells.⁶³ Spontaneous bacterial peritonitis has been reported.^{80,81}

Other sites of infection include the heart, central nervous system, and skin. Although rare, *Brucella* endocarditis is the most feared complication and accounts for 80% of deaths from brucellosis.^{82,83} Central nervous system infection usually manifests itself as chronic meningoencephalitis, but subarachnoid hemorrhage and myelitis also occur. Guillain-Barre syndrome has

TABLE 9-2
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.

been associated with acute neurobrucellosis, and involvement of spinal roots has been noted on magnetic resonance imaging.^{84,85} A few cases of skin abscesses have been reported.

Diagnosis

A thorough history with details of likely exposure (eg, laboratories, animals, animal products, or environmental exposure to locations inhabited by potentially infected animals) is the most important diagnostic tool. Brucellosis should also be strongly considered in the differential diagnosis of febrile illness in troops who are presumed to have been exposed to a biological attack. Polymerase chain reaction and antibody-based antigen-detection systems may demonstrate the presence of the organism in environmental samples collected from an attack area.

When the disease is considered, diagnosis is based on clinical history, bacterial isolation from clinical samples, biochemical identification of the organism, and 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 arthralgia."⁸⁶ Handling specimens for cultivation of *Brucella* poses a significant hazard to clinical laboratory personnel.⁸⁷⁻⁹⁰ Rapid detection of the organism in clinical samples using polymerase chain reaction–enzyme-linked immunosorbent assays (ELISA) or real-time polymerase chain reaction assays may eventually prove to be the optimal method for identification of these infections.⁹¹ According to the Centers for Disease Control and Prevention's 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;
- 4-fold 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.⁸⁶

Although several serologic techniques have been developed and tested, the tube agglutination test remains the standard method.⁹² 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 into monomers detects IgG antibody. A titer of 1:160 or higher is considered diagnostic. Most patients already have high titers at the time of

clinical presentation, so a 4-fold rise in titer may not occur. IgM 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 IgG) antibody titers has been associated with persistent disease or relapse.⁹³ Serum testing should always include dilution to at least 1:320 because 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. 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, which will allow for screening and confirmation of brucellosis in one test.^{94,95}

In addition to serologic testing, diagnosis should be pursued by microbiologic culture of blood or body fluid samples. If nonautomated 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, cultures should be incubated for at least 10 days with blind culture at 7 days.⁹⁶ The samples should be subcultured in a biohazard hood because it is extremely infectious. The reported frequency of isolation from blood varies from less than 10% to 90%; *B melitensis* is said to be more readily cultured than *B abortus*. A recent study indicated that BACTEC (Becton Dickinson Diagnostic Instrument Systems, Sparks, Md) Myco/F lytic medium, 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.²⁴ Culture of bone marrow may increase the yield and is considered superior to blood cultures.⁹⁷ In addition, direct fluorescent antibody tests under development may offer a method of rapidly identifying these organisms in clinical specimens (Figure 9-2). The case classification of "probable" is defined as a clinically compatible case that is epidemiologically linked to a confirmed case or 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.⁹⁸

Treatment

Brucellae are sensitive in vitro to a number of oral antibiotics and to intravenous/intramuscular 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 for *Brucella* (Table 9-3) and the standard procedures for in-vitro testing were established. These breakpoints and procedures were published in the new CLSI (NCCLS) guidelines in September–October 2005.⁹⁹ Therapy with a single drug has resulted in a high relapse rate; therefore, combined regimens should be used whenever possible.⁹⁸ A 6-week regimen of doxycycline at 200 mg per day administered orally, with the addition of streptomycin at 1 gram per day administered intramuscularly for the first 2 to 3 weeks, is effective therapy in adults with most forms of brucellosis.¹⁰⁰ However, a randomized, double-blind study using doxycycline plus rifampin or doxycycline plus streptomycin demonstrated that 100 mg of oral doxycycline twice daily plus 15 mg/kg body weight of oral rifampin once daily for 45 days was as effective as the classical doxycycline plus streptomycin combination, provided these patients did not have evidence of spondylitis.¹⁰¹ A 6-week oral regimen of both rifampin at 900 mg per day and doxycycline at 200 mg per day should result in nearly 100% response and a relapse rate lower than 10%.¹⁰² Several studies,^{100,103-105} however, suggest that treatment with a combination of streptomycin and doxycycline is more successful

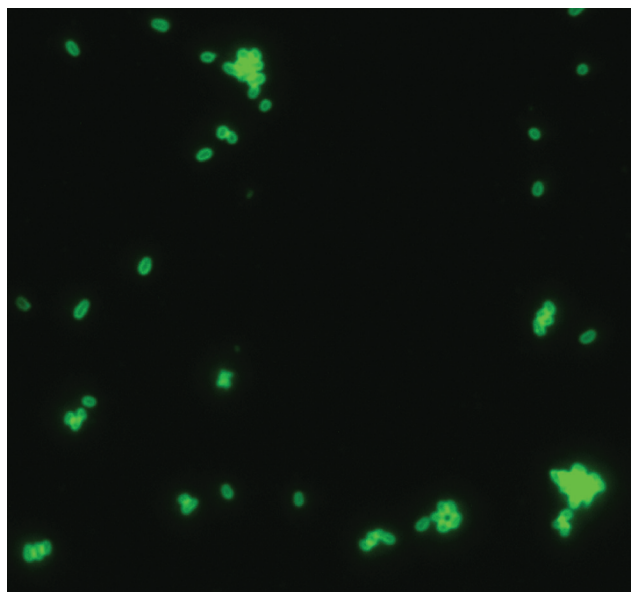


Fig. 9-2. Direct fluorescent antibody staining of *Brucella abortus*.

Photograph: Courtesy of Dr John W Ezzell and Terry G Abshire, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

TABLE 9-3

BRUCELLOSIS MINIMUM INHIBITORY CONCENTRATION BREAKPOINT RANGES

Antimicrobial	Minimum Inhibitory Concentration Range ($\mu\text{g}/\text{mL}$)
Azithromycin	0.25 – > 64
Chloramphenicol	0.5 – 4
Ciprofloxacin	0.25 – 8
Streptomycin	1 – 16
Tetracycline	0.03 – 0.5
Doxycycline	≤ 0.015 – 1
Gentamicin	0.5 – 4
Rifampin	< 0.12 – 2
Levofloxacin	< 0.06 – 4
Trimethoprim – Sulfamethoxazole	0.25 – 2

Data sources: (1) Patel J, Heine H. Personal communication from Clinical Laboratory Standards Institute (CLSI, formally known as National Committee for Clinical Laboratory Standards or NCCLS) June 2005 Guideline Meeting. (2) Patel J, et al. *J Clin Microbiol*. Publication pending.

and may result in less frequent relapse than treatment with the combination of rifampin and doxycycline. Although it is a highly effective component of therapy for complicated infections, streptomycin has the 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.⁷⁹ Fluoroquinolones in combination with rifampin have demonstrated efficacy similar to the doxycycline-rifampin regimen and may replace it because of potential doxycycline-rifampin interactions.¹⁰⁶⁻¹⁰⁹

Endocarditis may best be treated with rifampin, streptomycin, and doxycycline for 6 weeks. Infected valves may need to be replaced early in therapy.¹¹⁰ However, if patients do not demonstrate congestive heart failure, valvular destruction, abscess formation, or have a prosthetic valve, therapy with three antibiotics—(1) tetracycline or doxycycline, plus (2) rifampin, plus (3) aminoglycoside or trimethoprim/sulfamethoxazole for a mean duration of 3 months—may be effective.¹¹¹ 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 under 8 years old.¹¹²

The Joint Food and Agriculture Organization–World Health Organization Expert Committee recommends treating pregnant women with rifampin.¹⁰² In the case of a biological attack, the organisms used may be resistant to these first-line antimicrobial agents.

Medical officers should 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 (see Chapter 22) for a discussion of the biosafety levels that are used at the US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Md. Chemoprophylaxis is not generally recommended for possible exposure to endemic disease.

In the event of a biological attack, the M40 mask

(ILC Dover, Frederica, Del) should adequately protect personnel from airborne brucellae because 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.¹¹³ There is no commercially available vaccine for humans.

SUMMARY

Brucellosis is a zoonotic infection of large animals, especially cattle, camels, sheep, and goats. Although humans can acquire *Brucella* organisms by ingesting contaminated foods (oral route) or slaughtering animals (percutaneous route), the organism is highly infectious by the airborne route; this is the presumed route of infection of the military threat. Laboratory workers commonly become infected when cultures are handled outside a biosafety cabinet. Individuals presumably infected by aerosol have symptoms indistinguishable from patients infected by other routes: fever, chills, and myalgia are most common, occurring in more than 90% of cases.

Because the bacterium disseminates throughout the reticuloendothelial system, brucellosis 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. ELISAs are more sensitive and specific for brucellosis but have not been validated for standard laboratory use. 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 a 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 10

Q FEVER

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INTRODUCTION

HISTORY

MILITARY RELEVANCE

INFECTIOUS AGENT

- Disinfection
- Pasteurization
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DISEASE

- Epidemiology
- Pathogenesis
- Infection (Coxiellosis) in Animals
- Clinical Disease in Humans

DIAGNOSIS

- Serology
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TREATMENT

PROPHYLAXIS

SUMMARY

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INTRODUCTION

Q fever was discovered in Australia and in the United States before the outbreak of World War II. In Australia the disease was common in slaughterhouse workers and farm workers,¹ and it persists as an occupational problem.² This zoonotic disease is nearly worldwide and the etiologic agent, *Coxiella burnetii*, has a broad host range. Acute Q fever, although rarely life-threatening, can be temporarily incapacitating. Humans usually contract the disease by inhaling barnyard dust contaminated after parturition by infected

animals. A single microorganism is sufficient to cause infection. The infectious particle is extremely resistant to environmental degradation. Acute disease is not accompanied by unique symptoms. Therefore, Q fever must be considered in the differential diagnosis when a history of animal contact is established. Rarely, acute Q fever progresses to chronic Q fever, a debilitating, life-threatening infection that is difficult to treat. Because of its high infectivity and stability in the environment, *C burnetii* is listed as a Category B biothreat agent.

HISTORY

In 1933 a disease of unknown origin was first observed in slaughterhouse workers in Queensland, Australia. Patients presented with fever, headache, and malaise. Serologic tests for a wide variety of possible etiologic agents were negative.¹ Because the disease had an unknown etiology, it was given the name Q fever (for query). The infection was shown to be transmissible when blood and urine from patients elicited a febrile response after injection into guinea pigs. The infection could be passed to successive animals. Unfortunately, no isolate could be obtained after culture on bacteriological media, and the etiologic agent was thought to be a virus.

About this time, ticks were being collected in western Montana as part of an ongoing investigation into Rocky Mountain spotted fever. Ticks collected from the Nine Mile Creek area caused a febrile response when placed onto guinea pigs. The infection could be passed to successive guinea pigs through injection of blood.³ Examination of inflammatory cells from infected guinea pigs revealed rickettsia-like microorganisms, although the disease in guinea pigs was not spotted fever.⁴ A breakthrough in cultivating this agent occurred with the discovery that it would grow in yolk sacs of fertilized hens' eggs.⁵ Although the microorganism was demonstrated to be infectious, the disease it

caused was unknown. In Australia, however, a disease was identified, but it had an unknown etiology.

In Montana a researcher was infected while working with the Nine Mile isolate, and guinea pigs could be infected by injecting a sample of the patient's blood. At the same time, infected mouse spleens were sent from Australia to the United States. In a remarkable mix of serendipity and science, it was confirmed that the agent causing Q fever and the Nine Mile isolate were the same by demonstrating that guinea pigs previously challenged with the Nine Mile isolate were resistant to challenge with the Q fever agent.⁶ The conclusion could also be made that ticks transmitted Q fever. Although initially named *Rickettsia diaporica*⁷ and *Rickettsia burnetii*,⁸ the microorganism was given the name *C burnetii* in 1948 in honor of Dr Cox and Dr Burnet, who made important contributions regarding propagation and isolation of this agent.⁹

Investigations of Q fever soon established that *C burnetii* was prevalent in slaughterhouses and hazardous in the laboratory, and also could be spread by aerosol.^{10,11} The successful culture of the Q fever organism in chicken embryos proved to be a fortuitous breakthrough for advances in Q fever research, as well as for other rickettsial organisms.¹² Q fever has been identified in over 50 countries.¹³

MILITARY RELEVANCE

An atypical pneumonia, similar to Q fever, was noted in German soldiers in Serbia and southern Yugoslavia during World War II.¹⁴ The agent causing "balkengrippe" was not confirmed by laboratory testing, but the clinical and epidemiological features of the illness described were most consistent with Q fever. Hundreds of cases were observed in German troops in Italy, Crimea, Greece, Ukraine, and Corsica. Five Q fever outbreaks were also noted in American troops in Europe during the winter of 1944 and the spring of 1945.¹⁴ Cases usually occurred in troops occupying

farm buildings recently or concurrently inhabited by farm animals.¹⁵ However, cases also occurred in the absence of close contact with livestock. At an airbase in southern Italy, 1,700 troops became infected, presumably as a result of infected sheep and goats being pastured nearby.¹⁶

More recent Q fever cases in military service members have also occurred. An acute Q fever outbreak associated with a spontaneous abortion epidemic in sheep and goats occurred in British troops deployed in Cyprus, American airmen in Libya, and French

soldiers in Algeria, causing 78 cases of illness.^{14,17,18} Q fever outbreaks were also reported in Swiss and Greek soldiers and Royal Air Force airmen.¹⁴ Q fever has been identified in American military personnel in the Persian Gulf War. One case of meningoencephalitis associated with acute Q fever was reported in a soldier who recently returned from the Persian Gulf.¹⁹ Subsequent

serologic testing in the author's laboratory identified three additional acute seroconversions in soldiers of the same battalion. These reports underscore the necessity of considering the possibility of Q fever in service members having symptoms consistent with a Q fever and a recent history of exposure to livestock that may harbor *C burnetii*.

INFECTIOUS AGENT

C burnetii is an obligate intracellular pathogen of eukaryotic cells and replicates only within the phagolysosomal vacuoles of host cells, primarily macrophages. Growth does not occur on any axenic medium. During natural infections, the organism grows to high titer in placental tissues of goats, sheep, and possibly cows.^{20,21} This microorganism is routinely cultured in chicken embryo yolk sacs and in cell cultures,²² and it can also be recovered in large numbers within spleens of experimentally infected mice and guinea pigs.²² Growth is slow, with a generation time longer than 8 hours.²³

The microorganism usually grows as a small coccobacillus, approximately 0.8 to 1.0 μm long by 0.3 to 0.5 μm wide. Like other gram-negative microorganisms, *C burnetii* possesses a lipopolysaccharide (LPS), although the Gram stain reaction is variable.^{24,25} LPS is important in virulence and is responsible for the antigenic phase variation seen in this organism.^{26,27} *C burnetii* can display LPS variations similar to the smooth-rough LPS variation in *Escherichia coli*.²⁶ Bacterial isolates from eukaryotic hosts have a phase I (smooth) LPS character, which can protect the organism from microbicidal activities of the host. As those isolates are passed in yolk sacs or other nonimmunocompetent hosts, the phase I LPS character of the bacterial population gradually changes to the phase II (rough) form. Phase I microorganisms are virulent, and phase II microorganisms are avirulent in immune competent hosts.

The developmental cycle features small, compacted cell types within mature populations growing in animal hosts.²⁸ These forms, called small cell variants (SCVs), are responsible for the organism's high infectivity, as well as its capability to survive relatively extreme environmental conditions; its chemical resistance; and its resistance to desiccation, heat, sonication, and pressure.²⁹ The large cell variants (LCVs) are probably the metabolically active cells of this organism. The SCV and LCV are antigenically different.³⁰ Transition between SCV and LCV does not involve classical phase variation, which refers to LPS structure, but can be accompanied by changes in the expression of surface protein.

Coxiella is an obligate intraphagolysosomal parasite with acid-activated metabolism, presumably because most of its transport mechanisms required for import of required nutrient substrates from the vacuole envi-

ronment function in a pH range of 4.0 to 5.5. Purified organisms incubated without any host fractions or cells require an acid pH to transport or metabolize either glucose or glutamate.³¹ However, in-vitro growth under acidic conditions has not resulted in axenic growth, although protein synthesis can occur. Growth in the harsh phagolysosomal environment shows that this microorganism has coping strategies. The coping mechanism, although undefined, may involve the production of oxygen scavengers.³² An iron/manganese superoxide dismutase has been demonstrated, and genetic sequencing has also revealed a copper-zinc dismutase.³³ Because *C burnetii* is susceptible to reactive oxygen and nitrogen intermediates produced in response to infection by the host cells,³⁴ the microorganism's primary strategy for surviving within host cells is likely avoiding host cell activation. That phase I *C burnetii* does not activate human dendritic cells,³⁵ and that *C burnetii* LPS does not activate host antimicrobial responses via Toll-like receptor 4, are evidence to support this strategy.³⁶

Disinfection

Ten percent household bleach did not kill the organisms during a 30-minute exposure.³⁷ Likewise, exposure to 5% Lysol, 2% Roccal, or 5% formalin for 30 minutes did not inactivate *C burnetii*.³⁷ The organism was inactivated within 30 minutes by exposure to 70% ethyl alcohol, 5% chloroform, or 5% Enviro-Chem.³⁷ (The latter chemical, a formulation of two quaternary ammonium compounds, is known as Micro-Chem Plus and is available through National Chemical Laboratories, Philadelphia, Pa.) Formaldehyde gas can also be an effective sterilizing agent when administered in a humidified (80% relative humidity) environment.³⁷

Pasteurization

The frequent presence of *C burnetii* in cow's milk led to the establishment of effective milk pasteurization procedures. Temperatures of 61.7°C for 20 minutes can kill the organisms in raw milk.³⁸ In the laboratory, aqueous suspensions of the microorganism are typically killed by treating at 80°C for 1 hour.

Irradiation

Gamma irradiation can be used to sterilize biological preparations. The amount of gamma irradiation that reduced infectivity by 90% was 8.9×10^4 rads for *C burnetii* suspended in yolk sacs and 6.4×10^4 rads for the purified specimen.³⁹ The sterilizing dose was calculated to be 6.6×10^5 rads. Typically an irradiation dose of 2.1×10^6 rads is used for sterilizing

serum samples. An important consideration is that useful biological specimens are not degraded after activation by irradiation. Gamma irradiation (2.1×10^6 rads) was shown to have no deleterious effect on the antibody-binding capacity of *C burnetii* antigen, the antigen-binding capability of anti-*C burnetii* antibody, the morphological appearance of *C burnetii* by electron microscopy, or the distribution of a major surface antigen.³⁹

DISEASE

Epidemiology

Q fever is a zoonotic disease that occurs worldwide. Of the variety of species that can be infected by *C burnetii*, humans are the only species to develop symptomatic disease. Human infections are primarily found in persons occupationally exposed, such as ranchers, veterinarians, and workers in meatpacking plants. Domestic ungulates, such as cattle, sheep, and goats, usually acquire and transmit *C burnetii*, and domestic pets (primarily cats) can be a primary source of human infection in urban environments.⁴⁰⁻⁴² Heavy concentrations of microorganisms are secreted in milk, urine, feces, and especially in parturient products of infected pregnant animals.⁴³ Because of the stability of this agent, dried, infectious particles in barnyards, pastures, and stalls can be a source of infection months later.⁴³ Infection is most commonly acquired by breathing infectious aerosols or contaminated dust.⁴⁴ Patients can also be infected by ingesting contaminated milk⁴⁵ and through the bite of an infected tick.³ Infection can also occur in individuals not having direct contact with infected animals, such as persons living along a road used by farm vehicles⁴⁶ or those handling contaminated clothing.^{47,48}

C burnetii is extremely infectious for humans. The infectious dose is estimated to be 10 microorganisms or fewer.⁴⁹ The route of infection may determine the clinical manifestations of the disease.⁵⁰ In most cases of infections acquired by ingesting the microorganism, acute Q fever is found primarily as a granulomatous hepatitis.⁵¹ However, in patients infected by the aerosol route, Q fever pneumonia is more common.⁵² The infectious doses have been shown to vary inversely with the length of the incubation period.⁵³ Person-to-person transmission has been reported, but is rare.⁵⁴ The rates of Q fever seropositivity vary. In Nova Scotia, where extensive seroepidemiological work has been done, 14% of tested human samples were positive.⁵⁵ Overall, the incidence of Q fever is underreported. For example, in Michigan, although the first two Q fever cases were not reported until 1984, a survey showed

that 15% of the general population surveyed and 32% of goat owners had serologic evidence of infection.⁵⁶ The incidence of reported Q fever is higher now than in the 1990s, partly because of improved surveillance and more accessible testing.

Researchers find it controversial whether bacterial strains causing chronic Q fever are fundamentally different from strains causing acute Q fever. Some evidence suggested a link between genetic structure and the disease type (chronic or acute),⁵⁷ but other researchers thought that host-specific factors were more important.⁵⁸ The lack of a good chronic Q fever animal model made it difficult to resolve the question. However, a recent genetic analysis showed that groupings based on allelic differences of 159 *C burnetii* isolates from chronic Q fever cases were never found associated with acute disease.⁵⁹ This observation strengthens the case that the disease course in humans can be related to the strain of the infecting microorganism.

Pathogenesis

Q fever is an acute, self-limited systemic illness that can develop into a chronic, debilitating disease. Pathogenesis of infection in human disease is not well defined. Studies with animal models show that after initial infection of the target organ, the microorganism is engulfed by resident macrophages and transported systemically. The acidic conditions within the phagolysosome allow cell growth. Eventually proliferation within the phagolysosome leads to rupture of the host cell and infection of a new population of host cells. In animal models, the spleen and liver and other tissues of the reticuloendothelial system appear to be most heavily infected, which is likely the case in human infection. Chronic Q fever cases can arise years after the initial presentation. Animals frequently remain infected over their lifespans, with outgrowth of the microorganism occurring during conditions of immunosuppression, such as parturition,⁶⁰ or in laboratory animals that have been immunosuppressed.⁶¹ One of the unresolved mysteries of Q fever is where the microorganism is "hiding out"

in the intervening time between recovery from human acute disease and the development of chronic disease. Another unresolved question is whether humans ever completely clear the microorganism after infection. *Coxiella* DNA has been found in the bone marrow of the majority of patients who had primary Q fever 12 years previously.⁶² Asymptomatic animals may also harbor the microorganism.⁶³

Infection (Coxiellosis) in Animals

Coxiellosis is a zoonosis that affects native and domestic animals. Animals are infected by biting ectoparasites, primarily ticks, and by inhaling infectious particles.⁶⁴ Nursing calves can also be infected via their mother's milk—over 90% of dairy herds in the northeastern United States were found to be infected with *C burnetii*, based on surveillance of bulk milk samples.⁶⁵ Pasteurization of milk products decreases the risk of human infection. Infected animals generally appear to be asymptomatic, except for a rise in the rate of spontaneous abortions.⁶⁶ Domestic ruminants are the primary source of infection for humans. Eradication of *Coxiella* infection in animal populations is difficult because infection rarely causes symptoms. Unlike in humans, infection in animals does not cause pathological changes in the lungs, heart, or liver. The site most often affected is the female reproductive system, primarily the placenta, where damage is minimal. However, infection results in shedding vast quantities of organisms into the environment, which becomes a source of infection for other animals and humans.

Sheep have been a source of infection at medical research institutions, where animals used in neonatal research have caused Q fever in humans.⁶⁷⁻⁶⁹ However, unlike cattle and goats that tend to remain chronically infected,⁷⁰ sheep likely do not shed the organisms into the environment over a long period.^{64,71,72} Therefore, *Coxiella* infection in sheep might be a transient infection with a spontaneous cure, similar to most Q fever cases in humans.⁶⁴ Abortion is seen more often in infected sheep and goats than in cows.⁷³

Clinical Disease in Humans

The majority of human *C burnetii* infections are asymptomatic, especially among high-risk groups, such as veterinary and slaughterhouse workers, other livestock handlers, and laboratory workers.⁷⁴ The vast majority of the overt disease cases are acute Q fever. Fatalities in acute Q fever cases are rare, with fewer than 1% of cases resulting in death.¹ The incubation period can last a few days to several weeks, and the severity of infection varies in direct proportion to the infectious

dose.^{53,75} There are no characteristic symptoms of Q fever, but certain signs and symptoms tend to be more prevalent. Fever, severe headache, and chills are the symptoms most commonly seen. Fever usually peaks at 40°C and lasts approximately 13 days.⁷⁶ Fatigue and sweats are also frequently found.⁷⁷ Cough, nausea, vomiting, myalgia, arthralgia, chest pain, hepatitis, and occasionally, splenomegaly, osteomyelitis, and meningoencephalitis are also associated with acute Q fever.^{19,77} Blood tests show a normal white blood cell count, although thrombocytopenia or mild anemia may be present.⁷⁸ The erythrocyte sedimentation rate is frequently elevated.⁷⁹ Neurological symptoms, such as hallucinations, dysphasia, hemi-facial pain, diplopia, and dysarthria, have been described in an outbreak of acute Q fever.⁷⁸ The duration of symptoms increases with age.⁷⁶

Pneumonia is a common clinical presentation of acute Q fever.⁸⁰ Atypical pneumonia is most frequent, and asymptomatic patients can also exhibit radiologic changes that are usually nonspecific and can include rounded opacities and hilar adenopathy.^{40,81} Infection can also cause acute granulomatous hepatitis with corresponding elevations of the aspartate transaminase and/or alanine transaminase.⁷⁷ Elevations in levels of alkaline phosphatase and total bilirubin are seen less commonly.

Chronic Q fever is rarer, but also results in more deaths than acute Q fever. Patients with prior coronary disease or patients immunocompromised because of disease, such as AIDS, or therapy, such as immunosuppressive cancer therapy or antirejection therapy after organ transplant, are more at risk for developing chronic Q fever.^{82,83} Endocarditis, primarily of the aortic and mitral valves,⁸⁴ is the most common manifestation of chronic Q fever; although chronic hepatitis⁸⁵ and infection of surgical lesions⁸⁶ have been seen. Approximately 90% of Q fever endocarditis patients have preexisting valvular heart disease.⁸⁷ Of those acute Q fever patients with cardiac valve abnormalities, as many as one third develop endocarditis.⁸⁸ Patients with chronic Q fever lack T-cell responses, resulting in an immune response inadequate to eradicate the microorganism. This immunosuppression of host cellular immune responses is caused by a cell-associated immunosuppressive complex.⁸⁹ This complex may cause immunosuppression by stimulating the production of prostaglandin E2 and high levels of tumor necrosis factor, which may also have deleterious effects on the host.⁹⁰⁻⁹² Patients with chronic Q fever also have an increase in interleukin 10 secretion.⁹³ Suppression of host immunity may allow persistence of the microorganism in host cells during the development of chronic Q fever. Other pathological effects of chronic Q fever include the presence of circulating immune complexes, resulting in glomerulonephritis.⁹⁴

DIAGNOSIS

Serology

Q fever is difficult to distinguish because it lacks characteristic features. Diagnosis is usually based on clinical symptoms, a history of exposure to animals, and serologic testing. Although specific cellular immune responses may be suppressed in acute Q fever cases, humoral immune responses appear to continue unabated during infection.⁹⁵ Therefore, clinicians frequently encounter situations where a presumptive diagnosis of acute Q fever, based on nonspecific signs and serology, warrants a diagnosis of acute Q fever leading to therapeutic intervention.

The two antigenic forms of *C burnetii* that are important for serologic diagnosis of Q fever are the phase I (ie, virulent microorganism with smooth LPS [S-LPS]) and phase II (ie, avirulent microorganism with rough LPS [R-LPS]) whole-cell antigens.^{96,97} Determining antibodies against phase I and phase II *C burnetii* can help distinguish acute and chronic Q fever.⁹⁵ Infection of humans produces characteristic serologic profiles by various antibody tests. Although the complement fixation assay is generally regarded as the most specific serologic assay for Q fever, the indirect fluorescent antibody assay, the microagglutination assay, and the enzyme immunoassay can provide positive results earlier in the course of an infection.⁹⁸ Most diagnostic laboratories use either the indirect fluorescent antibody assay or enzyme immunoassay (Table 10-1). Both tests are sensitive and specific.⁹⁹ The indirect fluorescent antibody assay is generally used when equipment or space is limited or when small numbers of samples are tested. An advantage of the indirect fluorescent antibody assay is the ability to use phase I and phase II antigens unpurified from their yolk sac growth medium. The enzyme immunoassay is highly sensitive, easy to perform, has great potential adaptability for automation, and can be applied in epidemiological surveys.¹⁰⁰ A disadvantage is the requirement for a more highly purified cellular antigen for enzyme im-

munoassay. Such purified antigens are not usually commercially available.

Patients with acute Q fever may be distinguished from patients with chronic Q fever based on serologic results. In sera from acute Q fever patients, the magnitude of antiphase II titers exceeds those of antiphase I titers (Table 10-2).⁹⁵ However, in chronic Q fever patients, the antiphase I titers exceed those of anti-phase II titers, and patients with chronic Q fever endocarditis can have high levels of serum IgA.

Culture

Bacterial culture is not recommended for routine diagnosis of Q fever because of the difficulties and hazards associated with this agent. However, in research settings, the isolation and characterization of new strains can result in significant contributions to the phylogenetic study of the genus. Two basic methods are used to isolate *C burnetii* from clinical specimens: propagation of the microorganisms (1) in cell culture monolayers¹⁰¹ and (2) in rodents.²² In the "shell vial" technique, a eukaryotic cell monolayer is infected with patient tissues free of contaminants, and the presence of *C burnetii* is detected by fluorescent antibody methods or polymerase chain reaction (PCR). Results obtained using this technique are subjective and should not be the basis for making clinical decisions, predicting patient prognosis, or determining the presence of microorganisms in environmental samples.

Isolation of *C burnetii* from clinical samples can also be accomplished by injection of tissue homogenates into immunocompetent animals, such as mice.²² With this technique, crude estimates of bacterial number in the infected tissues can be made by diluting and injecting samples because only one infective microorganism is required for growth (resulting in seroconversion) in an animal host.¹⁰² The high infectivity and low mortality caused by infection increase the chances

TABLE 10-1
ASSAYS FOR THE SERODIAGNOSIS OF Q FEVER

Serologic Tests	Advantages	Disadvantages
Indirect fluorescent antibody	Can use unpurified diagnostic antigens	Inconvenient to test large numbers of sera
Enzyme-linked immunosorbent assay	Can evaluate large numbers of sera; used in epidemiological surveys	Requires highly purified diagnostic antigens

TABLE 10-2
SEROLOGIC DIAGNOSIS OF Q FEVER

Magnitude of Serologic Titers	Diagnosis
Antiphase II titer > antiphase I titer	Acute Q fever
Antiphase II titer < antiphase I titer	Chronic Q fever

of a successful isolation. Furthermore, contaminants found associated with tissues generally do not pose a problem for successful isolation because the host immune response should facilitate clearance of those microorganisms. Animals injected with homogenized infected tissues are bled at weekly intervals, and spleen homogenates from antibody-positive mice are injected into a new set of mice to allow the microorganisms

TREATMENT

Although it is not bactericidal, doxycycline is the recommended treatment for human acute Q fever.¹⁰⁷ The recommended dose for treating acute disease in adults is 100 mg doxycycline, twice daily.¹⁰⁷ However, doxycycline or tetracyclines alone are not sufficient for treating chronic Q fever; drug combinations are needed, especially when endocarditis is present. One of the most efficacious treatments is doxycycline plus hydroxychloroquine.¹⁰⁸ Q fever endocarditis patients generally receive 18 months of therapy with doxycycline, 100 mg twice daily, and chloroquine, 200 mg three times daily.¹⁰⁷ Quinolones can also be used for those who cannot tolerate chloroquine. For these patients, 3 years of therapy with doxycycline, 100 mg twice daily, and ofloxacin, 200 mg three times daily, is recommended.¹⁰⁷ The long duration is recommended because relapses

to propagate in the host in pure culture. After two to four animal passages, spleen cell suspensions are injected into embryonated eggs, and a *C burnetii* isolate is purified from the infected yolk sacs. Isolation of the Q fever etiologic agent is performed at research institutions engaged in studying the infectious agent and is unnecessary for diagnosing a case of Q fever in patients.

C burnetii can be identified in clinical samples, in infected cell cultures, or in infected lab animals by PCR.¹⁰³⁻¹⁰⁵ The most useful PCR targets are those that use the insertion sequence IS1111.¹⁰⁶ Each *C burnetii* Nine Mile Creek strain chromosome contains at least 19 copies of this sequence, and every *C burnetii* isolate tested so far has multiple copies of this element. Human leukocytes obtained from citrated or EDTA blood can be used for determining the presence of *C burnetii*.⁸⁰ *C burnetii* DNA was identified in the sternal wound of a chronic Q fever endocarditis patient by PCR.⁸⁶

have occurred when the latter regimen was stopped.¹⁰⁸ Hydroxychloroquine probably enhances the efficacy of the doxycycline by making the phagolysosome alkaline, which restricts *Coxiella's* acidophilic metabolism.¹⁰⁹ Yeaman and Baca have reviewed unsuccessful results with single treatments of doxycycline and chloramphenicol for human endocarditis.¹¹⁰ Recently, clarithromycin showed promise in acute Q fever clinical trials.¹¹¹ Strains of the microorganism that are resistant to antibiotics have been isolated.¹¹²

Evaluating antibiotic susceptibility of *C burnetii* isolates has been difficult because conventional methods cannot be used. An improved method has recently been developed using real-time PCR to determine bacterial replication in cells cultured in the presence and absence of antibiotics.¹¹³

PROPHYLAXIS

Control of *C burnetii* infection depends on stimulating a cell-mediated immune response, as is typical of microorganisms that grow intracellularly inside host cells.¹¹⁴ Laboratory experiments have shown that stimulation of macrophage antimicrobial mechanisms by T-cell gamma interferon production leads to control of infection.^{115,116} Passive transfer of antibodies did not control infection.¹¹⁷ In addition, pretreating *C burnetii* with specific antibodies before infection also failed to control intracellular replication.¹¹⁸

An efficacious Q fever vaccine was developed and available for human vaccination only a few years after discovery of the etiologic agent. This preparation

was rather crude, consisting of formalin-killed and ether-extracted *C burnetii* containing 10% yolk sack, but was effective in protecting human volunteers from disease after aerosol challenge.¹¹⁹ The phase of the microorganism is important in efficacy of the vaccine. In the early studies, the antigenic nature of the vaccine was not known. More recent vaccines for Q fever are prepared from phase I microorganisms because those preparations are 100 to 300 times more potent than phase II vaccines.¹²⁰ Improved purification methods were eventually developed to exclude egg proteins and lipids. Vaccine efficacy of these more highly purified preparations was demonstrated in

human volunteers.¹²¹ Although this and other early phase I cellular vaccines were efficacious, their use was occasionally accompanied by adverse reactions at the vaccination site, including induration or the formation of sterile abscesses or granulomas.¹²² Previously infected or previously vaccinated individuals were at risk for developing these adverse reactions.¹²² Approximately 3% of persons vaccinated for the ninth and tenth time developed severe persistent reactions.¹²³ The development and use of a skin test to exclude immune individuals from being vaccinated¹²⁴ resulted in a dramatic decrease in the incidence of adverse reactions after vaccination. Currently, skin testing is used to assess the potential for developing adverse vaccination reactions, although some laboratories also measure the level of specific antibodies against *C burnetii*.¹²⁵ Only individuals testing negative are vaccinated. Cellular *C burnetii* vaccines currently in use are safe and efficacious if the recipients are not immune before vaccination.

The most tested Q fever vaccine is Q-Vax (CSL Limited, Parkville, Victoria, Australia), a formalin-killed, phase I cellular vaccine that is produced and licensed for use in Australia.¹²⁵ In Australian studies, this vaccine has been 100% effective in preventing clinical Q fever in occupationally at-risk individuals, with the duration of protection exceeding 5 years.¹²⁵ However, the vaccine cannot be administered without prior determination of immunity. A similar product, which is not licensed, is administered as an Investigational New Drug. This vaccine is available through the US Army Medical Research Institute of Infectious Diseases for vaccinating at-risk persons in the United States.

Although attenuated microorganisms generally are not used as Q fever vaccines, a phase II attenu-

ated strain, designated M-44, was developed from the Greek "Grita" strain in the former Soviet Union.¹²⁶ This vaccine can produce an adverse reaction and caused myocarditis, hepatitis, liver necrosis granuloma formation, and splenitis in guinea pigs.¹²⁷ Human vaccinees did not develop antiphase I antibodies, and antiphase II levels were variable and at low titer.

Potential difficulties may be encountered in evaluating immunity before vaccination. Using serologic titer as an indicator of immunity may not eliminate the risk of adverse vaccination reactions because specific antibody titers decrease after acute infection¹²⁸ and may not accurately reflect the immune status of the individual. Performing skin tests is time consuming and expensive, and the test might be incorrectly applied or misinterpreted. Therefore, efforts are underway to develop safer Q fever vaccines that will pose a lesser risk if given to someone with preexisting immunity. Such a vaccine could eliminate the requirement for prevaccination screening of potential vaccinees while retaining vaccine efficacy. With only a single visit to a healthcare practitioner needed, vaccination would be simpler and less expensive. One candidate vaccine was made by extracting phase I whole cells with a mixture of chloroform and methanol. The residue after extraction (chloroform-methanol residue vaccine; CMR) did not cause adverse reactions in mice at doses much higher than doses of phase I cellular vaccine that caused severe adverse reactions.¹²⁹ Efficacy of CMR vaccine has been demonstrated in laboratory rodents, sheep, and nonhuman primates.¹³⁰⁻¹³³ Efficacious Q fever vaccines would benefit those occupationally at risk for Q fever, persons residing in areas endemic for Q fever, and soldiers or civilians who may be exposed due to a bioterrorist or biowarfare attack.

SUMMARY

Q fever is a zoonotic disease that is caused by the rickettsia-like organism *C burnetii*, which is important because of its exceptional infectivity. The disease is mainly transmitted by inhalation of infected aerosols, and a single organism may cause infection in humans. The disease is distributed worldwide, and the primary reservoir for human infection is livestock animals, particularly goats, sheep, and cattle. Contact with parturient animals or products of conception poses especially high risk because the organism is present in high numbers in this setting. The organism

is also resistant to pressure and dessication, and it may persist in a spore-like form in the environment for months.

Diagnosis is performed by serologic testing. Treatment of acute Q fever with tetracyclines is effective. Prevention is possible with a formalin-killed, whole-cell vaccine, but prior skin testing to exclude immune individuals is necessary to avoid the potential of severe local reactions. A Q fever vaccine is licensed in Australia, yet a similar product remains investigational in the United States.

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Chapter 11

SMALLPOX AND RELATED ORTHOPOXVIRUSES

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INTRODUCTION

Variola, the virus that causes smallpox, is one of the most significant bioterrorist threat agents. During the 20th century, smallpox is estimated to have caused over 500 million human deaths.¹ Yet 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; there are no animal reservoirs to reintroduce the virus into the human population. The impact of a smallpox virus attack in the human population would be even more catastrophic now than during the 20th century, because most vaccination programs were abandoned worldwide in the 1970s, the prevalence of immunosuppressed individuals has grown, and mobility, including intercontinental air travel, has accelerated the pace of viral spread. Smallpox virus is stable, highly infectious via the aerosol route, and highly transmissible from infected to susceptible persons, and it has a relatively long asymptomatic incubation period, making contact tracing difficult.³ Mathematical models of a variola reintroduction 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 such an attack.⁵ However, the 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.

Recent revelations that the former Soviet Union produced ton quantities of smallpox virus as a strategic weapon³ and conducted open-air testing of aerosolized variola on Vozrozhdeniye Island in the Aral Sea have increased the plausibility of variola being used as a bioterrorism agent.⁷ Considerable investment is being made in biopreparedness measures against smallpox and related orthopoxviruses, including emergency response plans for mass immunization and quarantine,⁸ as well as development of improved countermeasures such as new vaccines and antiviral drugs.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 monkeypox virus was weaponized by the former Soviet Union.¹⁰ 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 Democratic Republic of Congo, little morbidity was associated with infection. Over 50 human infections were documented in the United States as a result, demonstrating the public health importance of this agent and the potential bioterrorist threat.^{11,12}

AGENT CHARACTERISTICS

Classification

Poxviruses infect most vertebrates and invertebrates, causing a variety of 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 includes the human pathogens variola (Figure 11-1), monkeypox virus, and other species that infect humans such as cowpox and vaccinia viruses. Members of the *Orthopoxvirus* genus are mostly zoonotic pathogens, and a few of these viruses produce disease in humans (Table 11-1).

Morphology

Orthopoxviruses are oval, brick-shaped particles with a geometrically corrugated outer surface. Their size ranges from 220 nm to 450 nm long and 140 nm

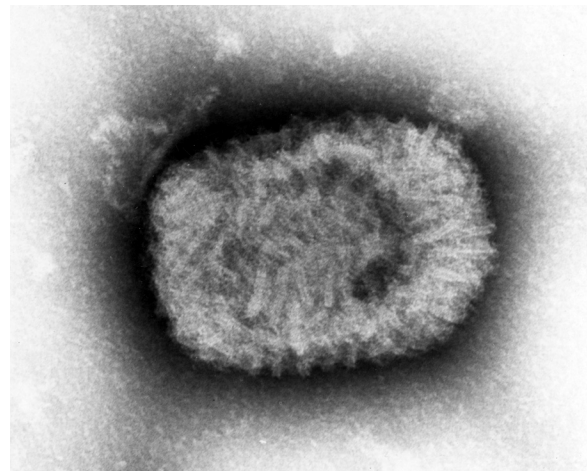


Fig. 11-1. A transmission electron micrograph of a tissue section containing variola viruses. Photograph: Courtesy of FA Murphy, University of Texas Medical Branch, Galveston, Texas.

TABLE 11-1
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 papular stomatitis virus	Cattle
	Orf virus	Sheep
	Pseudocowpox virus	Cattle
	Seal parapoxvirus	Seals
Parapoxvirus	Tanapox	Rodents (?)
	Yabapox virus	Monkeys (?)
Molluscipoxvirus	Molluscum contagiosum virus	None

to 260 nm wide. The outer envelope consists of a lipoprotein layer embedding surface tubules and enclosing a core described as biconcave because of an electron microscopy fixation artifact. 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. Between the palisade layer and the outer envelope are two oval masses known as the lateral bodies (Figure 11-2). Two infectious forms of orthopoxviruses (described next) result from the replication cycle.

Phylogenetic Relationships

The evolutionary relationships among the poxviruses have been facilitated by the recent availability of complete DNA sequences for over 30 species. Phylogenetic analysis reveals that variola and camelpox viruses are more closely related to each other than any other members of the genus, and vaccinia is most closely related to cowpox virus strain GRI-90.^{13,14} 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. Yet vaccination prevents monkeypox. Minor modifications to the camelpox virus genome might result in a virus with variola attributes. 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.

As anticipated from the genomic homologies, members of the *Orthopoxvirus* genus are antigenically related. Serum absorption and monoclonal antibody studies have identified cross-reacting and species-specific neutralizing antigens.¹⁵ Nine neutralizing epitopes have been identified among the intracellular

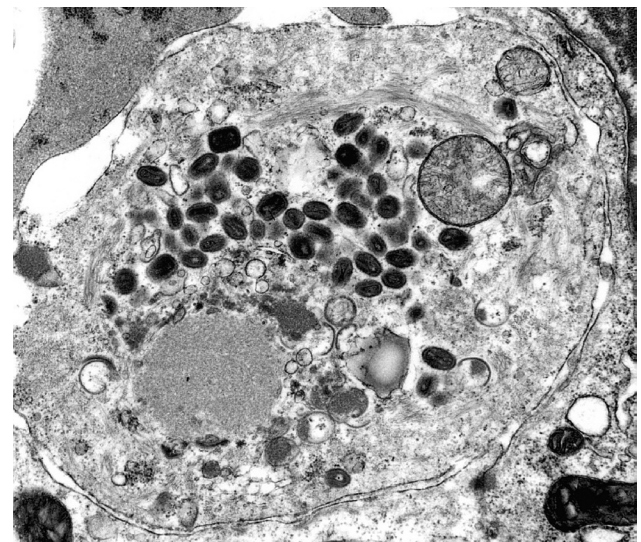


Fig. 11-2. Thin section of smallpox virus growing in the cytoplasm of an infected chick embryo cell of infected person. Intracellular mature virions (brick-shaped) and immature virions (spherical) are visible. Magnification is approximately $\times 25,000$.

Photograph: Courtesy of FA Murphy, University of Texas Medical Branch, Galveston, Texas.

mature virion (IMV) particles of different species of orthopoxviruses¹⁶; additional epitopes, believed to be critical in protection against infection in vivo, exist on extracellular enveloped viral particles.^{17,18} Viral envelope proteins are important in protective antibody responses: envelope antigens were absent from virion suspensions used for inactivated smallpox vaccines that proved to be ineffective.^{19,20}

Replication

Orthopoxvirus genomes are linear, double-stranded DNA approximately 200 kb long. The genomes encode about 176 to 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, and 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.²¹ 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 11-3).

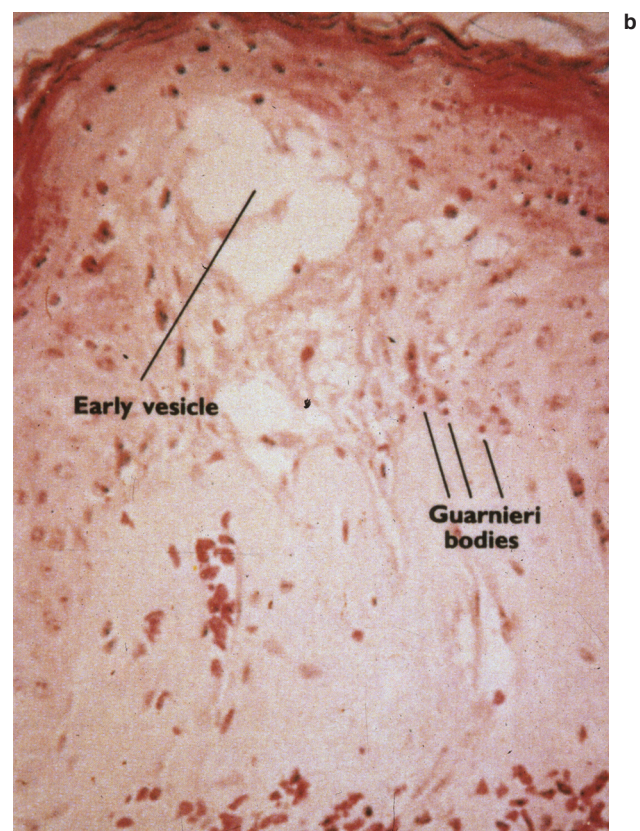
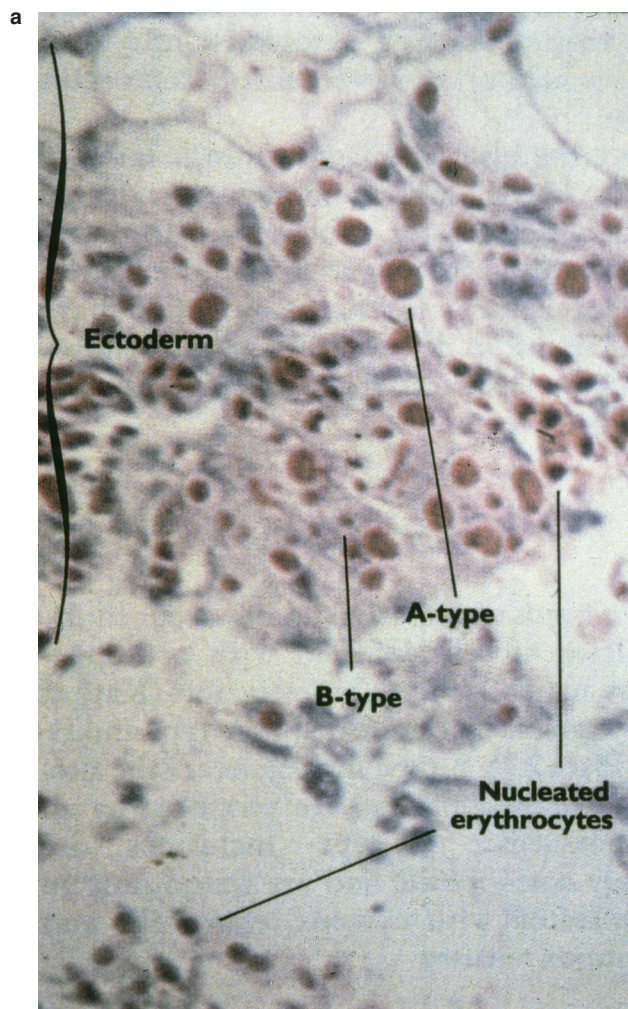


Fig. 11-3. Cytoplasmic inclusion bodies in cells infected with orthopoxviruses. (a) B-type (pale-red, irregular) inclusion, or Guarnieri, bodies, and A-type (large eosinophilic, with halo) inclusion bodies in ectodermal cells of the chorioallantoic membrane, in a pock produced by cowpox virus. A number 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.

Viral replication begins with attachment of viral particles to the host cell surface, most likely through cell receptors, and involves expression of early, intermediate, and late genes.²¹ Initial uncoating occurs during entry, followed by synthesis of early mRNAs, which are translated to facilitate further uncoating and transcription of intermediate mRNAs. Intermediate mRNAs, in turn, are translated to allow transcription of the late mRNAs. The late mRNAs are translated into structural and nonstructural proteins of the virions. These 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 brick-shaped infectious IMVs. IMVs are infectious only when they are released by cell lysis. IMV particles, which can acquire a second membrane from an early endosomal component to form the intracellular enveloped virion (IEV), migrate to the cell surface via microtubules and fuse with the cell membrane to form cell-associated virions (CEVs). CEVs induce polymerization of actin to form filaments that affect the direct transfer of CEVs to adjacent cells. If CEVs become dissociated from the cell membranes, they are called extracellular enveloped virions (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 the host cells and immune processes.^{21,23} 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.²⁴

Pathogenesis

Most knowledge about smallpox pathogenesis is inferred from animal studies of mousepox,^{25,26} rabbitpox,²⁶ and monkeypox^{27,28} in their respective hosts, and from vaccinia in humans. Studies using primates infected with variola²⁹ corroborate these findings and lend further insight into human smallpox and monkeypox infections. In both natural and experimental infections, the virus is introduced via the respiratory tract, where it first seeds the mucous membranes, including membranes of the eye, and then passes into local lymph nodes. The first round of replication occurs in the lymph nodes, followed by a transient viremia, which seeds tissues, especially those of the reticuloendothelial system,

including regional lymphatics, spleen, and tonsils. A second, brief viremia transports the virus to the skin and to visceral tissues immediately before the prodromal phase. In humans, the prodrome is characterized by an abrupt onset of headache, backache, and fever, and usually sore throat resulting from viral replication in the oral mucosa. Characteristic skin lesions develop following viral invasion of the capillary epithelium of the dermal layer. The virus may also be present in urine and conjunctival secretions.³⁰ At death, most visceral tissues contain massive virus concentrations.

In a review of all pathology reports published in English over the past 200 years,³¹ 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 might have caused a degree of compromise, but liver failure was not usually the proximate cause of death.

Much of the pathogenesis of smallpox remains a mystery because of the limited tools that were available when it was an endemic disease. Detailed analysis of the pathophysiology of the disease course using the monkeypox and variola primate models and in comparison with limited clinical and pathology data from human smallpox victims suggests a role for dysregulation of the immune response involving 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 probably associated with organ dysfunction and multisystem failure. Immunohistochemistry studies showing the distribution of viral antigens as well as electron microscopy evidence of the replicating virus correlated with pathology in the lymphoid tissues, skin, oral mucosa, gastrointestinal tract, reproductive system, and liver. Apoptosis was a prominent observation in lymphoid tissues, with a striking loss of T cells observed. The cause of this widespread apoptosis remains unknown. However, strong production of proinflammatory cytokines at least in part likely contributed to the upregulation of various proapoptotic genes. The strong upregulation of cytokines may also have 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. In human populations, however, the occurrence of hemorrhagic smallpox was approximately 1% to 3% of the total cases observed.

From these recent studies of variola and monkeypox virus infection in primates, the “toxemia” described by clinicians for human smallpox² may be fundamentally related to the processes underlying septic shock.³² 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 gen-

erally 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.³³

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.³⁴⁻³⁶ During the American Civil War, allegations were made about the use of smallpox as a biological weapon, although no definite evidence existed.^{37,38} In the years leading up to and during World War II, the Japanese military explored weaponization of smallpox 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 smallpox on a continuing basis well into the 1980s.^{10,39} The former Soviet Union also conducted open air testing of weaponized smallpox virus and demonstrated that infectious virus could drift 15 km downwind and infect humans.⁷

Although declared stocks of smallpox 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, 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.³⁹ The probability that such stocks exist 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 because of waning immunity following vaccinations in the distant past and immunosuppression resulting from HIV, cancer, organ transplants, and old age.³ Oth-

er 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.⁴⁰ The disease is 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 (TCID₅₀).^{27,41-43} Monkeypox virus, like variola, is relatively stable and can resist desiccation in both heat and cold.⁴⁴ The monkeypox virus also can grow 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.²⁸ Monkeypox virus may have already been weaponized by the Soviet military.¹⁰

Cowpox and buffalopox produce limited cutaneous disease in humans in natural infection.⁴⁵ Buffalopox, like cattlepox, may be essentially identical to vaccinia.⁴⁶ 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.^{28,47-51} Aerosol infection has the potential to produce more pronounced pulmonary disease.^{28,42,52} In addition, all orthopoxviruses share a significant amount of homology with variola and monkeypox.¹⁴ If the critical virulence factors for systemic human disease were found, then cowpox,

buffalopox, or other orthopoxviruses potentially could be genetically modified to express these critical factors. When designed as a weapon and delivered by aerosol, these viruses could have 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.⁵³ Camelpox virus is the closest relative of variola virus; the major difference between camelpox virus and variola strain Bangladesh-1975 genomes is four additional insertions, elongated inverted terminal repeats, and a small area of gene rearrangement present in camelpox virus.¹³ As with other orthopoxviruses, slight modifications in the camelpox virus genome might 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 may soon be technically feasible to create infectious variola using an oligonucleotide synthesizer, analogous to the recent demonstration for creation of the much simpler polio virus.⁵⁴

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.^{55,56} Whether these results represent findings unique to the virus-host model used or reflect a more general premise of enhanced virulence is unclear.^{57,58} 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.⁵⁹ Variola virus is infectious by aerosol,³ but natural airborne spread other than among close contacts is unusual.^{60,61} Approximately 30% of susceptible contacts became infected during the era of endemic smallpox,⁶² 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 the variola virus can be spread through airborne dissemination in conditions of low relative humidity.⁶³ 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 infection were greatly increased. Indirect transmission via contaminated bedding or other fomites was infrequent.⁶⁴ Some people in close contact with patients harbored virus in their throats without developing disease and may have been a means of secondary transmission.^{65,66}

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 viremia, which is followed by a rash.⁶⁷ The incubation period of smallpox averages 12 days (range 9–14 days). Those in contact with infected patients are quarantined for a minimum of 16 to 17 days following exposure.⁶⁷ Following infection via the respiratory route and replication in local lymph nodes, variola

virus disseminates systemically to other lymphoid tissues, spleen, liver, bone marrow, and lung. During this asymptomatic, prodromal period, variola virus can be recovered from the blood, but the yield is lower than later in the illness. Clinical manifestations begin acutely with malaise, fever, rigors, vomiting, headache, and backache; 15% of patients develop delirium. Approximately 10% of light-skinned patients exhibit an erythematous rash during this phase. After 2 to 3 more days, an enanthem appears concomitantly with a discrete rash about the face, hands, and forearms. Because of 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.⁶⁸ These respiratory secretions are the most significant but not the sole means of virus transmission. Following subsequent eruptions on the lower extremities, the rash spreads centrally during the next week to the trunk. Lesions quickly progress from macules to papules and eventually to pustular vesicles (Figure 11-4). Lesions are more abundant on the extremities and face, and this centrifugal distribution is an important diagnostic feature. In contrast to the lesions seen in varicella, 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 on healing. Although variola titers in the throat, conjunctiva, and urine diminish with time,⁶⁷ virus can readily be recovered from



Fig. 11-4. 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.



Fig. 11-5. 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.

scabs throughout convalescence.⁶⁹ 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, the highly virulent, prototypical, and historically significant form of the disease, remained prevalent in Asia and parts of Africa during the 20th century. 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.⁷⁰ 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. 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.⁷¹ Three quarters of variola major cases were designated classic or ordinary type (see Figure 11-4). 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. 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, focal skin lesions that did not resemble

the classical variola exanthem (Figure 11-5). This syndrome caused 66% mortality in vaccinated patients and 95% mortality in unvaccinated patients. Fewer than 3% of smallpox patients developed hemorrhagic-type smallpox, which was accompanied by extensive petechiae (Figure 11-6), mucosal hemorrhage, and intense toxemia; death usually occurred before typical pox lesions developed.⁷² However, on occasions hemorrhagic smallpox also occurred in the classic type later in the disease. Both hemorrhagic-type and flat-type smallpox may have indicated underlying im-

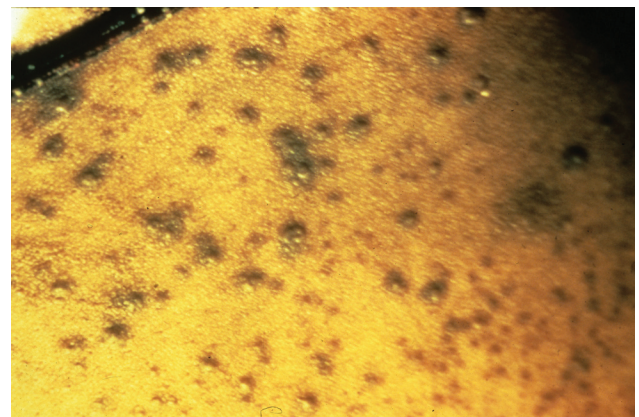


Fig. 11-6. Early hemorrhagic-type smallpox with cutaneous signs of hemorrhagic diathesis. Death usually intervened before the complete evolution of pox lesions. Reproduced with permission from Herrlich A, Munz E, Rodenwaldt E. *Die pocken; Erreger, Epidemiologie und klinisches Bild*. 2nd ed. Stuttgart, 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.

munodeficiency; hemorrhagic forms occurred more commonly in pregnant women and young children.⁷³ The modified type, which occurred typically but not exclusively in previously vaccinated individuals, was characterized by moderation of constitutional symptoms, typically reduced numbers of lesions, and 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.⁷¹ In actuality, the manifestations of variola infection fall along a spectrum, and classification is 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 and 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.⁷⁴ 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 fewer than 1% of cases. Disease during pregnancy precipitated high perinatal mortality, and congenital infection was also recognized.

Partial immunity caused by 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.⁷⁵ 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,⁷⁶ up to several decades in some individuals.¹⁸

Monkeypox

The clinical features of human monkeypox are classically described as being similar to those of smallpox.⁷⁷ 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.^{77,78} Lymphadenopathy is a common finding that differentiates monkeypox from smallpox. Lymphadenopathy, which has been documented in up to 83% of unvaccinated persons with monkeypox, 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.⁷⁷ In a series of 282 patients, the exanthema first appeared somewhere other than the face in 18% of the vaccinated patients; 31% of vaccinated patients had pleomorphic or "cropping" appearance of rash lesions, and 9.4% had centripetal distribution.⁷⁹ All of these features are inconsistent with a mimic of smallpox. Patients in the recent 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.^{80,81} None of the initial cases was suspected as a smallpox-like disease. A *sine eruptione* form of monkeypox has not been described, but the number of serologically diagnosed infections without consistent rash illness suggests that it is a possibility.⁸² A hemorrhagic form of human monkeypox has not been documented.^{83,84}

Complications of monkeypox are more common in unvaccinated persons and children.⁸⁵ During intensive surveillance in the Democratic Republic of the Congo 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.⁸⁶ Encephalitis was detected in at least one monkeypox case in the Democratic Republic of the Congo and in one of the cases in the US outbreak of 2003.^{79,81} 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.^{77,86-88} 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 1997.⁴⁰ No fatalities occurred among 78 suspected cases in the recent US outbreak.⁸⁰ The presence of comorbid illnesses, such as measles, malaria, or diarrheal disease, may have a significant impact on mortality in children.⁸⁵ 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.⁴⁵ Baxby, Bennett, and Getty reviewed 54 cases of cowpox infection with a detailed discussion of clinical manifestations.⁸⁹ 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.^{89,90} Three of these four described cases included a history of atopic dermatitis, indicating a risk of increased severity of disease analogous to vaccinia.

Buffalopox infection in humans has not been extensively described. Limited data suggest that human infection usually occurs on the hands and consists of inflamed and painful pustular lesions progressing through a Jennerian evolution.⁹¹⁻⁹³ Regional lymphadenopathy and fever can accompany local disease.⁹³

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 Web site (<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.

Laboratory Diagnosis

Collection of appropriate specimens is paramount for accurate laboratory diagnosis of *Orthopoxvirus* infection. For virological diagnosis, specimens from

skin lesions are most important, because when viremia does occur in *Orthopoxvirus* infections, it is an early phenomenon.² Ideally, cutaneous tissue and blood are sent for diagnostic testing, with other samples being sent at the request of public health officials or experts in the field.⁸⁴ Detailed instructions for specimen collection can be found in the Department of Defense Smallpox Response Plan (<http://www.bt.cdc.gov/agent/smallpox/response-plan/index.asp>) or on the CDC Web site (<http://www.cdc.gov/ncidod/monkeypox/diagspecimens.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 marble-topped or yellow-topped serum separator tube (which is then centrifuged to separate serum) and in a purple-topped 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.⁹⁴

Many phenotypic and genotypical methods involving virological, immunological, and molecular approaches have been used to identify *Orthopoxvirus*.

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 11-3]) in tissue samples under light microscopy. These cytoplasmic inclusions are hematoxylinophilic, stain reddish purple with Giemsa stain, and contain Feulgen-positive material.⁹⁵ Microscopy alone cannot differentiate members of the *Orthopoxvirus* genus, yet the epidemiological setting can suggest which species is involved. The orthopoxviruses with pathogenicity for 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,⁹⁶ other monkey kidney cell lines, A549, 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.^{97,98} Electron microscopy reveals the unmistakable brick-like morphology of orthopoxviruses in thin sections of infected materials. Immunogold stains permit 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 However, significant serologic cross-reactivity exists between all the *Orthopoxvirus* species; therefore, species differentiation is not possible with conventional serologic assays. Techniques developed in the 1980s to detect monkeypox-specific antibodies are complex and considered unreliable by some experts.^{82,99} Although complement-fixation tests detect antibodies that disappear within 12 months of infection, other traditional techniques, such as immunofluorescence assay, radioimmunoassay, enzyme-linked immunosorbent assay (ELISA), hemagglutination-inhi-

bition and neutralization assay, detect immunoglobulin (IgG) antibodies that are persistent. Thus, differentiating antibodies due to acute infection from antibodies resulting from prior vaccination can be difficult with single specimens.

Immunofluorescence assays and ELISAs have been used to detect IgM in acute infection directed against cowpox and monkeypox, respectively.^{90,99} Because IgM seems to 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.⁸¹ More recently, a combination of T-cell measurements and a novel IgG ELISA was used to enhance epidemiological follow-up studies to this outbreak.^{100,101}

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^{102,103} and microarray genotyping¹⁰⁴ also provide high levels of specificity, and when combined with PCR, these approaches can offer high levels of sensitivity. Real-time PCR methods provide exquisite levels of sensitivity and specificity.¹⁰⁵ The basic concept behind 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, Calif), 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), Smart Cycler (Cepheid, Synntvale, Calif), LightCycler (Roche Diagnostics Corporation, Indianapolis, Ind), MJ Opticon (Bio-Rad, Hercules, Calif), RotorGene (Corbett Life Science, Sydney, Australia); RAPID (Idaho Technology, Salt Lake City, Utah); 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.

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. There are numerous commercial nucleic acid purification methods 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, 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 is gel analysis, in which PCR-amplified regions of the genome are separated on agarose gels by electrophoresis, and 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.^{98,106-108}

Large fragment PCR-RFLP (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.

Recently developed 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.^{105,109-111} Real-time PCR was one of the diagnostic techniques used in the investigation of the 2003 US monkeypox outbreak.⁸¹ Because of its sensitivity, rapidity, and ease, 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.⁷⁰ During a smallpox outbreak in Boston in 1752, over 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.¹¹²

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 causes 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. 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.⁷⁰ 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. Because of the risk of bioterrorism, smallpox vaccination in at-risk military personnel and civilian healthcare workers was resumed in 2003.^{113,114}

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 about 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 post-vaccinial encephalitis. Although rare, this condition was frequently fatal. Death occurred in approximately one in one million primary vaccinations.^{115,116} 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 over 450,000 military vaccinees.¹¹³ In this recent 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.¹¹⁴ A potential replacement vaccinia was prepared in massive quantities (> 300 million doses) by selection of plaque-purified progeny virus from the New York Board of Health strain, which was amplified in VERO cell cultures. This vaccine is more purified and free of adventitious agents in comparison with its predecessor, which was prepared on calf skin. Phase I safety and immunogenicity trials for ACAM 2000 indicate greater than 95% take rates and adverse events comparable to those of live vaccinia.¹¹⁷ Historically, live (replicating) vaccinia immunization has also been used as postexposure prophylaxis and is believed effective if administered within 4 days of exposure.

The recent immunization of modest numbers 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-10.¹¹⁸ 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 are immunized with vaccinia and challenged with monkeypox, Edghill-Smith has shown that vaccinia-specific B cells are critical for protection.¹¹⁹ Antibody depletion of B cells, but not CD4⁺ or CD8⁺ T cells, abrogated vaccinia-induced protection. Edghill-Smith has also shown that simian-immunodeficiency-virus-

compromised monkeys could withstand vaccinia 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 less than 300 mm.³

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 over 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.¹²⁰ 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 (Wyeth Laboratories, Marietta, Pa).¹²¹ Surprisingly, a single dose of MVA also protected when 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. Rhesus monkeys were used in a similar intravenous challenge model to evaluate a DNA vaccine strategy, a combination of four genes (L1R, A27L, A33R, and B5R) with promising results.¹²²

The smallpox vaccine used in the United States is Dried, Calf Lymph Type (Dryvax), a live-virus preparation of the New York Board of Health vaccinia strain prepared from calf lymph. The calf lymph is purified, concentrated, and lyophilized. The diluent for the vaccine contains 50% glycerin and 0.25% phenol in US Pharmacopeia sterile water, with no more than 200 bacterial organisms per milliliter in the reconstituted product (Polymyxin B sulfate, dihydrostreptomycin sulfate, chlortetracycline hydrochloride, and neomycin sulfate are used in the processing of the vaccine, and therefore small amounts of these antibiotics may be present in the final product).

Vaccination is performed with a bifurcated needle onto which the reconstituted vaccinia preparation has been drawn, using three intradermal jabs for immunologically naïve individuals (new vaccinees) or 15 jabs for prevaccinated individuals, 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 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." 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.

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 importations 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 over 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.¹²³ Multiple vaccinations are thought to produce more long-lasting immunity. Vaccination has been effective in preventing disease in 95% of vaccinees.¹²⁴ Vaccination also was shown to prevent or substantially lessen the severity of infection when given as 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);
- active 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 breast-feeding. A history of Darier's disease and household contact with active disease 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.¹²⁵

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,¹²⁶ generalized vaccinia, eczema vaccinatum, progressive vaccinia, postvaccinial central nervous system disease, and fetal vaccinia.⁶

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 (Vira-A, King Pharmaceuticals, Bristol, Tenn) is often recommended, although treatment of ocular vaccinia is not specifically approved by the Food and Drug Administration for either of these drugs. Most published experience is with use of vidarabine, but this drug is no longer manufactured.¹²⁷

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 affects individuals who are age 2 or older and 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 vaccinia 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, 67 symptomatic cases of myopericarditis were reported, for a rate of 1.2 per 100,000. 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 120,000 vaccinees.¹¹² 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.^{128,129}

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 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 other *Orthopoxvirus* diseases.² Despite a lack of hard evidence, postexposure vaccination is likely efficacious against other orthopoxviruses, and during the 2003 US monkeypox outbreak the CDC recommended vaccination of potentially exposed persons.⁸⁰

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

Antiviral drugs would be useful for treatment of orthopoxviral diseases including smallpox and monkeypox, as well as adverse effects associated with vaccination. The only antiviral drug available for treating orthopoxviruses is cidofovir, which may be offered under emergency use protocols maintained by both the Department of Health and Human Services and the Department of Defense.

The elaborate replication strategy of poxviruses offers a number of potential targets for therapeutic intervention.¹³⁰ Although inhibition of viral replication may be necessary to halt the pathogenic disease course, it may not be sufficient—it may also be necessary to reverse the effects of the mounting damage that increasingly appears to be the result of a cytokine storm, which accounts for the “toxicity” of systemic orthopoxvirus infection.²⁹ In this regard, cytokine antagonists developed to treat bacterial sepsis and other conditions may play a role in effective management of smallpox- and monkeypox-infected patients.

Initial studies to identify effective antiviral agents for orthopoxviruses tested drugs developed for other viruses that share molecular targets with poxviruses.¹³¹ The effort to discover effective drugs against DNA viruses initially focused on treatment of herpesviruses 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. The failure of acyclovir to inhibit cytomegalovirus was 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.¹³² Cidofovir is licensed

for treatment of cytomegalovirus-associated retinitis under the trade name Vistide (Gilead Sciences Inc, Foster City, Calif), and may inhibit the cytomegalovirus DNA polymerase, a target shared with the poxviruses. Cidofovir also may inhibit the activity of the proofreading exonuclease, leading to error-prone DNA synthesis during poxvirus replication. Cidofovir has been demonstrated to protect monkeys against severe disease in both the monkeypox and authentic smallpox primate models, when administered within 48 hours of intravenous exposure to the virus.¹³³ 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 cidofovir administration would not significantly increase the healthcare burden. Because cidofovir has been associated with nephrotoxicity, primarily in dehydrated patients, careful attention to fluid management is important, and patient hydration and coadministration of probenecid is required.

Oral formulations of cidofovir analogues with better bioavailability and lower toxicity, designed to overcome the lack of an active transport pathway for unmodified cidofovir into cells, are under development.¹³⁴ Cidofovir requires bolus dosing to allow drug entry into cells by pinocytosis; however, bolus dosing results in transiently high concentrations in the kidney. The primary design paradigm for oral formulations is the creation of a lipid mimic that allows drugs to enter cells via the chylomicron pathway.¹³⁵ This formulation dramatically reduced transient drug levels in the kidney and eliminated nephrotoxicity in toxicology studies using mice. However, an oral formulation of cidofovir is not available for human use.

The first drug used to empirically treat progressive vaccinia and smallpox was Marboran, a compound of the class of N-aminomethyl-isatin-beta-thiosemicarbazones. As with most early treatment strategies, controlled clinical trials were not reported, and recent studies show that Marboran was only capable of inhibiting replication by 80% at maximum tolerated concentration in VERO cells.¹³⁶ Through combinatorial chemistry, potent and more selective compounds have now been discovered and are in preliminary testing.¹³⁷ A number of essential viral enzymes have been targeted using a homology-based bioinformatics approach, such as that used to develop a structural model of vaccinia virus I7L proteinase. A unique chemical library of 51,000 compounds was computationally queried to identify potential active site inhibitors.¹³⁸ A subset of compounds was assayed for toxicity and ability to inhibit vaccinia replication, and a family was identified with 50% minimal inhibitory concentrations of 3

to 12 μ M. Alternatively, a high-throughput screening approach using cowpox virus evaluated a collection of over 250,000 compounds and identified several potent lead structures for optimization and evaluation against vaccinia, monkeypox, and variola viruses. 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(1H)-yl)-benzamide} was identified and is under development. ST-246 is both potent ($EC_{50} = 0.010 \mu$ M), selective ($CC_{50} > 40 \text{ mM}$), and active against multiple orthopoxviruses, including monkeypox, camelpox, cowpox, ectromelia (mousepox), vaccinia, and variola viruses in vitro and monkeypox, variola, cowpox, vaccinia, and ectromelia in vivo.

Alternative approaches include peptide mimetics of IFN- γ that play a direct role in the activation of STAT 1 alpha transcription factor.¹³⁹ These mimetics do not act through recognition of the extracellular domain of the IFN- γ receptor; rather, they bind to the cytoplasmic domain of the receptor chain and thereby initiate the cellular signaling. The authors hypothesize that mimetics would bypass the poxvirus virulence factor B8R protein that binds the intact IFN- γ and would prevent interaction with its receptor. Experimentally, these mimetics, but not intact IFN- γ , inhibited replication of vaccinia in BSC-40 cells. Thus these mimetics can avoid the B8R virulence factor and have potential activity against poxviruses in vivo.

Gleevec (Novartis Pharmaceuticals Corporation, East Hanover, NJ), a drug licensed for use in chronic myeloid leukemia, has been shown to block the egress of vaccinia virus from infected cells.¹⁴⁰ Smallpox virus includes an epidermal-growth-factor-like domain that targets human Erb-1, inducing tyrosine phosphorylation of certain host cell substrates, thereby facilitating viral replication. Poxviruses migrate to the cell membrane via the polymerization of actin tails to produce EEV, which facilitates viral dissemination. The authors reason that low molecular weight inhibitors of Erb-1 kinases might function as antiviral agents. CI-1033, one such inhibitor, blocked variola replication in BSC-40 and Vero cells, primarily at the level of secondary viral spreading. CI-1033 protected mice exposed to a lethal vaccinia challenge via the aerosol route. In conjunction with a monoclonal antibody directed against L1R, CI-1033 cleared the mice's lungs of virus within 8 days. Gleevec is also a small molecule that inhibits the Abl-1 family of tyrosine kinases, thereby inhibiting the release of EEV from infected cells. Gleevec inhibited the vaccinia virus spread from the mouse peritoneum to the ovaries and protected the mice from all lethal intranasal challenge. The advantage of Gleevec over other tyrosine kinase inhibitors such as CI-1033 is that it is already approved for human use. The potential

success of Gleevec suggests that strategies that block key host signaling pathways have merit and augment the approaches that target classical viral replication enzymes. An alternative approach to inhibiting the polymerization of actin, which in turn inhibits the propulsion of viral particles along actin filaments toward the cell membrane, is small interfering RNA directed against the Arp2/3¹⁴¹ complex.

ST-246 is a new drug that is orally available and is currently in phase I human safety studies. Based on activity in multiple small animal models, oral ST-246 was evaluated in a recent USAMRIID study with a variola virus-cynomolgus monkey model of classical smallpox that closely resembles human disease.¹⁴² The placebo group demonstrated typical disease with greater than 1,250 pox lesions and 33% mortality. Oral gavage with ST-246 began 24 hours after infection, when bone marrow, spleen, some lymph nodes, and liver have greater than 10^8 genomes per gram and all tissues have 10^4 to 10^6 per gram, eliminated disease as judged by complete lack of lesion formation, the best predictor of smallpox disease severity in man, and no significant clinical or laboratory findings. Virus levels in blood did not increase over pretreatment levels (10^6 /mL), and virus was cleared in 6 days versus 16 days for placebo based on historical data. ST-246 was next evaluated using the authors' monkeypox virus-cynomolgus monkey model of classical smallpox that also closely resembles human disease. The placebo-treated group demonstrated typical disease with greater than 1,500 pox lesions and 100% mortality. Oral gavage treatment with ST-246 began 1 day after infection, when bone marrow, spleen, some lymph nodes, and liver have greater than 10^7 genomes per gram and all tissues have 10^5 to 10^6 per gram, and

eliminated disease as judged by complete lack of lesion formation and no significant clinical or laboratory findings. Virus levels in blood did not increase over pretreatment levels and virus was cleared in 4 days versus 16 days for placebo or intravenous cidofovir based on historical data. Oral gavage treatment with ST-246 began 3 days after infection, when bone marrow, spleen, some lymph nodes, and liver have greater than 10^8 genomes per gram and all tissues have greater than 10^6 per gram, eliminated disease as judged by complete lack of lesion formation in 2 of 3 monkeys and less than 5% of control lesions in 1 of 3 that did not progress, and no significant clinical or laboratory findings resulted. Virus levels in blood did not increase over pretreatment levels and virus was cleared in 6 days versus 16 days for placebo. ST-246 has been granted fast track investigational new drug status and has not shown toxicity in phase I human single oral dosing at 2,000 mg and is now in repeated dosing studies.¹⁴²

Lastly, treatment strategies may be developed to target the toxemia or clinical manifestations of smallpox. In particular, modulation of the systemic immune response to orthopox infection, specifically the prevention of organ damage caused by vascular leakage and fibrin deposition, may provide a useful therapeutic target. Uncontrolled or inappropriate immune responses can contribute to multiple organ failure and death; in this respect the "toxemia" associated with fatal orthopox infections resembles severe sepsis. Several treatment strategies for targeting the manifestations of septic shock,¹⁴³ such as activated protein C and inhibitors of the tissue factor pathway,¹⁴⁴ are under consideration for testing in the nonhuman primate model for smallpox.

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, recent revelations have shown that the Soviets continued developing smallpox for biowarfare into the 1980s. The Soviet Union is dissolved and its offensive program 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. Because 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 orthopox 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 for treatment of orthopoxvirus infections and safer vaccines; however, much work remains. There is still no approved treatment for smallpox, and the new safer vaccines remain unlicensed and unavailable.

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Chapter 12

ALPHAVIRUS ENCEPHALITIDES

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SUMMARY

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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 (WEE) virus was isolated in the San Joaquin Valley in California in 1930¹; eastern equine encephalitis (EEE) virus was isolated in Virginia and New Jersey in 1933^{2,3}; and Venezuelan equine encephalitis (VEE) virus was isolated in the Guajira Peninsula of Venezuela in 1938.⁴ By 1938 it was clear that EEE and WEE viruses were also natural causes of encephalitis in humans.⁵⁻⁷ Naturally acquired human infections with VEE virus 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. EEE is the most severe of the arboviral encephalitides, with case fatality rates of 50% to 70%, and neurological sequelae are common in survivors. WEE virus appears to be less neuroinvasive but has a pathology similar to that of EEE in patients with encephalitis. In contrast, severe encephalitis resulting from VEE virus is rare in humans except for children. In adults, the VEE virus usually causes an acute, febrile, incapacitating disease with prolonged convalescence.

The three viruses are members of the *Alphavirus* genus of the family *Togaviridae*. As with most of the alphaviruses, VEE, EEE, and WEE are transmitted by mosquitoes and maintained in cycles with various vertebrate hosts. Environmental factors that affect the interactions of the relevant mosquito and reservoir host populations control the natural epidemiology of these viruses. Of the 32 viruses classified within this group, VEE, EEE, and WEE are the only viruses regularly associated with encephalitis. Although these encephalitic strains are restricted to the Americas, as a group, alphaviruses have worldwide distribution and include other epidemic human pathogens. Among those pathogens, chikungunya virus (Asia and Africa), Mayaro virus (South America), O'nyong-nyong virus (Africa), Ross River virus (Australia), and Sindbis virus (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. VEE virus 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.⁹ Before vaccines were developed, most

laboratories working with VEE virus reported disease among their personnel. In one incident reported in 1959 at the Ivanovskii Institute in Moscow, in the former Soviet Union, at least 20 individuals developed disease within 28 to 33 hours after a small number of vials containing lyophilized virus were dropped and broken in a stairwell.^{10,11} The ability of aerosolized EEE and WEE viruses to infect humans is less certain, although the possibility is implied from animal studies. Additionally, WEE viruses are less commonly studied in the laboratory than VEE virus, and fewer human exposures may explain the lower incidence of laboratory-acquired infections.

Perhaps as a consequence of their adaptation to dissimilar hosts in nature, the alphaviruses replicate readily and generally to high titers, in a wide range of cell types and culture conditions. Virus titers of 1 billion infectious units per milliliter are not unusual, and the viruses are stable in storage and in a variety of laboratory procedures. Because they can be easily manipulated in the laboratory, these viruses have long served as model systems to study various aspects of viral replication, pathogenesis, induction of immune responses, and virus-vector relationships. As a result, the alphaviruses are well described, and their characteristics are well defined.^{12,13}

The designers of offensive biological warfare programs initiated before or during World War II¹⁴ recognized that the collective in-vitro and in-vivo characteristics of alphaviruses, especially the equine encephalomyelitis viruses, lend themselves well to weaponization. Although other encephalitic viruses could be considered as potential weapons (eg, the tick-borne 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 VEE and EEE viruses, as well as the inherent difficulties of inducing efficient mucosal immunity, confound defensive vaccine development.

The equine encephalomyelitis 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 km². 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,^{15,16} yet, in theory, it could also be used to increase the weaponization potential of equine encephalomyelitis viruses.

HISTORY AND SIGNIFICANCE

Descriptions of encephalitis epizootics in horses thought to have been caused by EEE virus were recorded as early as 1831 in Massachusetts.¹⁷ However, it was not until the outbreaks of EEE in Delaware, Maryland, and Virginia in 1933 and 1934 that the virus was isolated. During a similar outbreak in North Carolina in 1935, birds were first suspected as the natural reservoir.¹⁸ The initial isolation of EEE virus from a bird¹⁹ and from *Culiseta melanura* mosquitoes,²⁰ the two major components of the EEE natural cycle, were both reported in 1951. Outbreaks of EEE virus have occurred in most eastern states and in southeastern Canada but have been concentrated along the eastern and Gulf coasts. Although only 211 EEE cases in humans were reported²¹ between 1938 and 1985, the social and economic impact of this disease has been larger than might be expected because of the high fatality rate, equine losses, extreme concern among individuals living in endemic areas during outbreaks, and the surveillance and mosquito-control measures required. Isolation of EEE virus from *Aedes albopictus* mosquitoes, which were recently introduced into EEE endemic areas in the United States, has heightened concern because of the opportunistic feeding behavior of these mosquitoes and their apparent high vector competence for EEE virus.²²

The initial isolation in 1930 of WEE virus from the brain tissues of a horse with encephalitis was made during a large and apparently unprecedented epizootic in California, which involved at least 6,000 horses with an approximate mortality of 50%.¹ Cases of human encephalitis in California were not linked to WEE 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 Saskatchewan and Manitoba in Canada, and affected large numbers of equines and humans. For example, it has been estimated that during 1937 and 1938, more than 300,000 equines were infected in the United States, and in Saskatchewan, 52,500 horse infections resulted in 15,000 deaths.^{23,24} 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%.²⁴

In the early 1940s, workers isolated WEE virus from *Culex tarsalis* mosquitoes²⁵ and demonstrated the

presence of specific antibody to WEE virus in birds,²⁶ suggesting that birds are the reservoirs of the virus in nature. The annual incidence of disease in both equines and humans continues to vary widely, which is indicative of an arthropod-borne disease. Significant epidemics occurred in 1952, 1958, 1965, and 1975.²⁴

VEE virus 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 EEE and WEE viruses isolated previously in the United States.^{4,27} Over the following 30 years, many VEE outbreaks were reported among horses, and humans became infected in large numbers in association with these epizootics.²⁸ Most of those infected recovered after suffering an acute, febrile episode, but severe disease with encephalitis and death also occurred, mostly in children and older individuals. Major epizootics occurred in Venezuela, Colombia, Peru, and Ecuador in the 1960s, apparently spreading to Central America in 1969.²⁹ These epizootics and previous ones were associated with costly and dire consequences, especially among rural people, who not only had the disease but also lost their equines, 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 1995.³⁰

Between active epizootics, it was not possible to isolate the equine virulent viruses. During the 1950s and 1960s, however, several other attenuated, antigenically different VEE strains were isolated from different geographical areas. These enzootic strains could be differentiated antigenically not only among themselves but also from the epizootic strains.³¹ Enzootic strains used different mosquito vectors than the epizootic strains³² and used rodents as reservoir hosts.³³ Many of the enzootic strains, however, proved equally pathogenic for humans.

Therefore, within 30 years of the initial isolation of the EEE, WEE, and VEE viruses, an accurate picture had emerged of their endemic and epidemic behavior, arthropod vectors, reservoir hosts, and the diseases produced. Although not yet 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 States³⁴ and elsewhere, and it was evident that the equine encephalomyelitis viruses were preeminent 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 destroyed¹⁴ 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.*³⁵

Continuing efforts within the defensive program in the 1960s and 1970s produced four vaccines for the encephalomyelitis viruses: live attenuated (TC-83) and formalin-inactivated (C84) vaccines for VEE, and formalin-inactivated vaccines for EEE and WEE. These vaccines are used under investigational new drug status for at-risk individuals, distributed under investigational new drug provisions, and recommended for use by any laboratory working with these viruses.⁹ Although these vaccines are useful, they have certain disadvantages (discussed later in this chapter), and second-generation vaccines are being developed.¹⁵

ANTIGENICITY AND EPIDEMIOLOGY

Antigenic and Genetic Relationships

The three American equine encephalitis virus complexes, VEE, EEE, and WEE, have been grouped with four additional virus complexes into the *Alpha-virus* genus based on their serologic cross-reactivity (Table 12-1).¹³ Analysis of structural gene sequences obtained from members of the VEE and EEE virus complexes confirms the antigenic classification and serves as another tool for classifying these viruses (Figure 12-1). The WEE virus complex, including Highlands J, Fort Morgan, and WEE viruses, is identified as recombinant viruses originating from ancestral precursors of EEE and Sindbis viruses and, therefore, falls into a unique genetic grouping of alphaviruses.³⁶⁻³⁹

Venezuelan Equine Encephalitis Virus Complex

The VEE virus complex consists of six closely related subtypes that manifest different characteristics with respect to ecology, epidemiology, and virulence for humans and equines (Table 12-2). The IA/B and C 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 equines. All epizootic strains are exotic to the United States and have been isolated from areas where virus occurs naturally.⁴⁰ Subtypes II, III, IV, V, and VI and varieties ID, IE, and IF are referred to as the enzootic strains.⁴¹⁻⁴⁶ 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. The enzootic viruses are commonly isolated in specific ecological habitats, where they circulate in transmission cycles primarily involving rodents and *Culex*

mosquitoes of the *Melanoconion* subgenus.⁴⁷⁻⁴⁹ Infection of equines with some enzootic subtypes leads to an immune response capable of protecting the animals from challenge with epizootic strains.⁵⁰ Limited data, acquired following laboratory exposures, suggest that cross-protection between epizootic and enzootic strains may be much less pronounced in humans.⁵¹⁻⁵³

Eastern Equine Encephalitis Virus Complex

The EEE virus complex consists of viruses in two antigenically distinct forms: (1) the North American and (2) the South American variants.⁵⁴ The two forms can be distinguished readily by hemagglutination inhibition and plaque-reduction neutralization tests.^{54,55} All North American and Caribbean isolates show a high degree of genetic and antigenic homogeneity. However, they are distinct from the South American and Central American isolates, which tend to be more heterogeneous and form three genetic clades that are readily distinguished from the monophyletic North American EEE viruses.^{56,57}

EEE is endemic to focal habitats ranging from southern Canada to northern South America. The virus has been isolated as far west as Michigan but is most common along the eastern coast of the United States between New England and Florida. Enzootic transmission of EEE virus 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, known as bridging vectors, such as members of the genera *Aedes* and *Coquilletidia*. Mosquito vectors belonging to *Culex* species may play a role in maintaining and transmitting South American EEE strains.⁵⁸

TABLE 12-1
ANTIGENIC CLASSIFICATION OF ALPHAVIRUSES

Antigenic Complex	Virus		
	Species	Subtype	Variety
Western Equine Encephalitis (WEE)	WEE		
	Y 62-33 Highlands J Fort Morgan Aura Sindbis	Sindbis Babanki Whataroa Kyzylagach	Ockelbo
Venezuelan Equine Encephalitis (VEE)	VEE	I	A-B
		I	C
		I	D
		I	E
		I	F
		II Everglades III Mucambo	Mucambo Tonate 71D-1252
Eastern Equine Encephalitis (EEE)	EEE	IV Pixuna V Cabassou VI AG80-663	North American South American
		Semliki Forest	
Semliki Forest	Semliki Forest Chikungunya	Chikungunya	Several Igbo ora
		O'nyong-nyong	
		Getah	
		Sagiyama	
Middelburg Nduma Barmah Forest	Middelburg Nduma Barmah Forest	Ross River	
		Mayaro	
		Una	

Adapted with permission from Peters CJ, Dalrymple JM. Alphaviruses. In: Fields BM, Knipe DM, eds. *Virology*. 3rd ed, Vol 1. New York, NY: Raven Press; 1990: 716.

Western Equine Encephalitis Virus Complex

Six viruses, WEE, Sindbis, Y 62-63, Aura, Fort Morgan, and Highlands J, comprise the WEE complex. Several antigenic subtypes of WEE virus have been identified, but their geographical distributions overlap.⁴⁰ Most of the members of the WEE complex are distributed throughout the Americas, but subtypes of Sindbis virus and its subtypes have strictly Old World distributions.¹³ The New World WEE complex viruses can be distinguished readily by neutralization

tests. In addition, WEE complex viruses isolated in the western United States (ie, WEE) are antigenically and genetically distinct from those commonly found in the eastern United States (ie, Highlands J).^{57,59} Sindbis virus is considered a member of the WEE virus complex based on antigenic relationships. However, sequence comparisons show that WEE, Highlands J, and Fort Morgan viruses are actually derived from a recombination event between ancestral Sindbis and EEE viruses. The structural domains of the recombinant viruses were derived from the Sindbis virus ancestor,



Fig. 12-1. 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.

and the nonstructural domains were derived from the EEE virus ancestor.^{57,60}

The most studied member of the WEE virus complex in terms of its epidemiology is the WEE virus itself. The virus is maintained in cycles involving passerine birds and the mosquito *C tarsalis*. Humans (and equines) become involved only tangentially and are considered to be dead-end hosts,⁶¹ indicating that they do not normally contribute to further spread of the virus. Recent studies have isolated WEE virus from male *Ae dorsalis* mosquitoes reared in the laboratory from larvae collected in salt marsh habitats,⁶² suggesting that vertical transmission (ie, direct transmission from one generation to the next) in mosquitoes may be an important mechanism for persistence and overwintering in endemic areas.

Epidemiology and Ecology

The evolution of the equine encephalitides in humans is closely tied to the ecology of these viruses in naturally occurring endemic foci. Recent evidence indicates that the relative genetic homogeneity of the EEE and WEE virus complexes 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. 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 VEE virus 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 significant genetic change.⁶³

Human involvement in the form of endemic and epidemic activity occurs most commonly following intrusion into geographical regions where natural transmission cycles are occurring or after perturbation of these cycles by environmental changes or the addition of other vectors.⁶⁴ The dramatic exception to this is epizootic VEE, 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 VEE make them efficient amplifying hosts, with the result that equine infections normally precede human infections by days to weeks.⁶⁵ Researchers suggest that it is the adaptation of these viruses for efficient replication in horses that leads to the emergence and efficient epidemic spread of disease.^{22,66} Medical personnel should view with some suspicion evidence of widespread human VEE infections outside of endemic areas in the absence of mosquito vectors or in the absence of equine disease; this combination of circumstances may indicate an unnatural release of virus into the environment.

Enzootic VEE virus subtypes, as described above, are maintained efficiently in transmission cycles involving mosquitoes belonging mainly to the subgenus *Melanoconion*. These mosquitoes often live in humid localities with abundant open spaces such as sunny, swampy pastures cut by slowly flowing streams. They are ground feeders, seldom found higher than 8 meters above ground, and prefer feeding on mammals rather than birds.⁶⁷ 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 VEE virus. After infection, these animals develop viremia of sufficient magnitude and duration to infect mosquitoes feeding on their blood.⁶⁸ Other animals, such as bats and certain birds, may play a secondary role.⁶⁹ Seroprevalence rates among human populations living in or near endemic VEE areas vary but can approach 100%, suggesting that continuous transmission occurs.⁶⁵ 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 camped only a few yards away showed no disease.^{70,71} The incidence of 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.⁷² After an epizootic in Guatemala and El Salvador, overall seroprevalence was estimated at 20%.⁷³

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 genetic drift from enzootic strains. Results from oligonucleotide fingerprinting and sequence analysis of I-D 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 I-D virus.^{74,75}

Although the genetic evidence indicates that genetic

drift 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 VEE vector *C (Melanoconion) taeniopus* is fully susceptible to both I-AB and I-E strains following intrathoracic inoculation. Orally exposed mosquitoes, however, are fully competent vectors of the enzootic strain, but they fail to develop disseminated infection or transmit epizootic virus.^{32,76} In the absence of genetic change, this virus–host interaction appears to be relatively stable. Mosquito resistance to epizootic strains of VEE virus is rare. Epizootic strains have been isolated from a large number of mosquito species, and many have been shown to be efficient vectors.⁷⁷ Thus, host switching from enzootic to epizootic vectors may be an important factor in the evolution of epizootic VEE strains. Researchers have suggested that emergence of epizootic strains may result from acquisition of mutations that allow for

TABLE 12-2

THE VENEZUELAN EQUINE ENCEPHALOMYELITIS COMPLEX

Subtype	Variety	Prototype Strain	Origin	Cycle	Disease in	
					Horse	Man
I	A/B	Trinidad donkey	Donkey (Trinidad) ¹	Epizootic	+	+
	C	P-676	Horse (Venezuela) ²	Epizootic	+	+
	D	3880	Human (Panama) ³	Enzootic	–	+
	E	Mena II	Human (Panama) ¹	Enzootic	–	+
	F	78V-3531	Mosquito (Brazil) ⁴	Enzootic	–	?
II (Everglades)		Fe3-7c	Mosquito (Florida) ⁵	Enzootic	–	+
III (Mucambo)	A	Mucambo (BeAn8)	Monkey (Brazil) ⁶	Enzootic	–	+
	B	Tonate (CaAn410-D)	Bird (French Guiana) ⁷	Enzootic	–	+
	C	71D-1252	Mosquitoes (Peru) ⁸	Enzootic	–	?
IV (Pixuna)		Pixuna (BeAn356445)	Mosquito (Brazil) ⁶	Enzootic	–	?
V (Cabassou)		Cabassou	Mosquito (French Guiana) ⁷	Enzootic	–	?
VI		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 September 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, Fla: 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 arthropodborne 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, Tex: 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. *Revista Argentina de Microbiología.* 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, Fla: CRC Press; 1989: 206.

transmission by abundant equiphilic mosquitoes. More specifically, adaptation to *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.⁶⁶ The introduction of mosquito species into previously unoccupied geographical ranges (eg, *Ae albopictus* into North America) may, therefore, offer the opportunity for epizootic strains to reemerge.

A major epizootic VEE outbreak occurred in the late 1960s and early 1970s. Epizootic virus first reached North America in 1966 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,⁷² 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, and the first recorded such epizootic, occurred in 1966 in Tampico, Mexico, involving approximately 1,000 equines.

By the end of 1969 and the beginning of 1970, the outbreak had expanded to such an extent that the Mexican government requested the TC-83 vaccine from the US Army through the US Department of Agriculture.⁷⁸ Despite the vaccination of nearly 1 million equines, 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 secretary of agriculture to declare a national emergency on July 16, 1971.⁷⁹ Subsequent immunization of over 2 million horses and unprecedented mosquito abatement efforts eventually stopped the epizootic before it spread from Texas. Epizootic VEE 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 12-1 and 12-2). 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 over 20 deaths reported. This outbreak



Fig. 12-2. 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 including using insecticides to reduce mosquito populations and prohibiting the transportation of equines from affected areas.

was caused by an IC strain of VEE virus. By sequence analysis, this strain proved to be essentially identical to a virus that caused an outbreak in Venezuela in 1963.³⁰ More recently, outbreaks of traditionally enzootic strains of VEE have occurred in Mexico and Central America. Unlike previously identified enzootic strains, these newly emerged strains appear to have increased virulence for humans. Genetic analysis confirms acquisition of mutations, which provides further evidence that emergence of epizootic strains may result from accumulation of genotypic changes in enzootic strains.^{80,81}

STRUCTURE AND REPLICATION OF ALPHAVIRUSES

Virion Structure

The alphavirus virion, a spherical particle approximately 60 to 65 nm in diameter, is typically composed of three different 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 a single species of capsid (C) protein (Figure 12-3). 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^{82,83} to form the short spikes on the surface of the virion. The glycoproteins are the primary targets of the neutralizing antibody response and are one of the determinants of tropism and virulence.⁸⁴⁻⁸⁶ Semliki Forest virus contains a third glycoprotein, E3, which is associated with the E1–E2 dimers on the virion

surface. With other alphaviruses, the E3 protein is shed from the infected cell and does not appear in the mature virion.

Viral Infection

The infection cycle is initiated when the glycoprotein spikes on the virion bind to receptors on the cell surface. The virus is initially localized to 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.⁸⁷ 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.

Genomic RNA

The viral genome, a positive-stranded 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).⁸⁸ 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 (cDNA) clone of an *Alphavirus* is also infectious, which allows genetic manipulation of these viruses. Mutations introduced into a cDNA clone by site-directed mutagenesis are reflected in the RNA transcribed from the altered clone and in the virus obtained from transfected cells. These procedures are being used to develop improved vaccines,¹⁵ but they could also be used to enhance specific characteristics required for weaponization.

Glycoprotein Synthesis

The *Alphavirus* genome contains two protein coding regions. The 5' proximal 7,500 nucleotides encode a 220,000-dalton precursor polypeptide, which is proteolytically processed to produce 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-stranded 45S RNA, identical in length to the genome RNA but of opposite polarity. The negative-stranded 45S RNA subsequently serves as a template for the synthesis of additional genomic RNA. The negative-stranded RNA is also used as a template for transcription of a capped and polyadenylated 26S subgenomic mRNA, which is identical to the 3' third of the genome. The 26S mRNA is translated to yield a precursor polypeptide that is proteolytically processed by cotranslational and post-

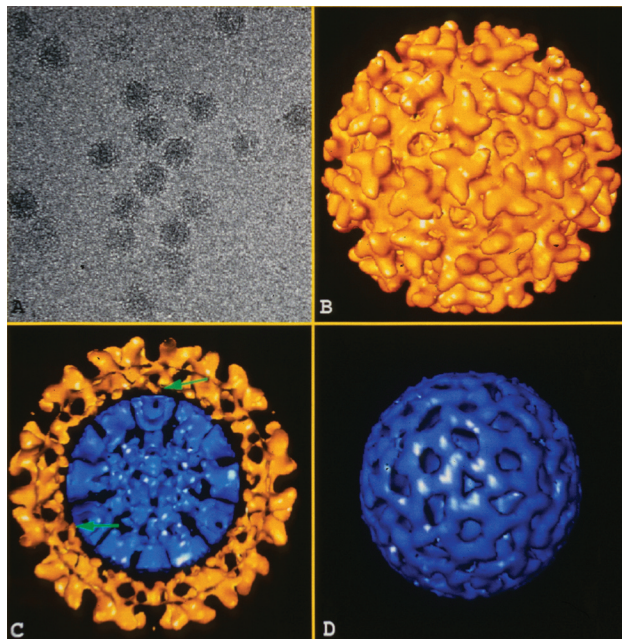


Fig. 12-3. Structure of an alphavirus. Shown is the three-dimensional reconstruction of Sindbis virus at 28 Å resolution from computer-processed images taken by electron cryomicroscopy. (a) The original electron micrograph shows virus particles in vitreous ice. (b) The surface view of the virus shows details of the 80 trimeric spikes, which are arranged in a T=4 icosahedron. Each spike protrudes 50 Å from the virion surface and is believed to be composed of three E1-E2 glycoprotein heterodimers. (c) The cross-sectional view shows the outer surface spikes (yellow) and the internal nucleocapsid (blue), composed of the capsid and viral RNA. The space between the spikes and the nucleocapsid would be occupied by the lipid envelope. The green arrows mark visible points of interaction between the nucleocapsid and transmembrane tails of the glycoprotein spikes. (d) The reconstructed capsid also exhibits a T=4 icosahedral symmetry. Computer models: Courtesy of Angel M Parades, Cell Research Institute and Department of Microbiology, The University of Texas at Austin, Austin, Texas. Similar but not identical versions of these computer models were published in Parades AM, Brown DT, Rothnagel R, et al. Three-dimensional structure of a membrane-containing virus. *Proc Natl Acad Sci U S A.* 1993;90:9095–9099.

translational cleavages to produce the viral structural proteins. The order of the structural proteins within the precursor is C-E3-E2-6K-E1.

As the 26S 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 continues, the polypeptide is translocated into the lumen of the endoplasmic reticulum, where oligosaccharides and fatty acids are added.⁸⁹ 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 6 K polypeptide probably serves as a signal for membrane insertion of the second glycoprotein, E1, and is subsequently cleaved from both E2 and E1 by signal peptidase.⁹⁰ A hydrophobic anchor sequence 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 E2 (PE2) and E1 interact to form multimeric complexes,⁹¹ which are then transported through the Golgi apparatus, where the final modifications of the oligosaccharide are made. The precursor pE2 is cleaved to the mature E2 and E3 glycoproteins soon after the glycoproteins

leave the Golgi apparatus,⁹² 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,⁹³ and in arthropod cells, budding also occurs at intracellular membranes.⁹⁴

In vertebrate cells, budding is initiated when intracellular nucleocapsids bind to the 30- to 40-amino acid cytoplasmic domain of the E2 glycoprotein,⁹⁵⁻⁹⁷ inducing the formation of a locally ordered array of glycoprotein spikes, which exclude 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 process of 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.⁹⁸ In arthropod cells, however, alphaviruses initially replicate to high titer 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 VEE, EEE, and WEE infections acquired by aerosol, which is the route of greatest biological defense concern, is unknown. Little is known of the pathogenesis even after natural vectorborne infections of humans, mainly because of limited autopsy material. Much of the information on VEE pathogenesis in humans is based on a histological review of 21 human fatalities from the 1962-1963 VEE epidemic in Zulia, Venezuela.⁹⁹ 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 animals¹⁰⁰⁻¹⁰⁸ 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 experimentally infected animals, were frequent histological findings

in these cases of severe human disease. Much of the understanding of the pathogenesis of VEE, EEE, and WEE has relied on animal studies. However, little work has been done in recent years with EEE and WEE viruses, and animal models have failed to recapitulate important characteristics of the human conditions. Recently, a hamster model for EEE, which appears to more closely resemble human EEE, has been developed and appears promising.¹⁰⁹ The pathogenesis of VEE virus infection, in contrast, has been extensively studied in animals, and the remainder of this section covers that subject.

The clinical and pathological responses of the host to VEE 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 have come from studies using rodent models challenged with the virulent Trinidad donkey (TrD) strain of VEE, an epizootic IA serotype virus, or its genetic clone V3000. A few nonhuman primate studies with monkeys have also been done. 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 VEE virus causes limited and reversible lesions to the lymphoid organs of mice and nonhuman primates,¹⁰¹⁻¹⁰⁵ but in guinea pigs and hamsters, it causes extreme and irreversible damage to those organs.^{106,107} As a result, in the guinea pig and hamster models, death occurs before the development of serious CNS disease.^{103,104} The host species and the route of administration of VEE virus 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.^{101,105,110,111} Nonhuman primates, however, reportedly 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.¹⁰⁰ Monkeys infected intranasally had more moderate inflammation, especially in the cortex and hypothalamus,¹¹² yet a Colombian epizootic strain of VEE given by the aerosol route caused severe clinical and pathological CNS signs and resulted in death in approximately 35% of rhesus monkeys.¹⁰² Both mice and cynomolgus monkeys challenged intracerebrally with TrD or related VEE strains developed severe and

lethal neurological signs with moderate to severe brain histopathology.^{112,113}

The mechanisms of neuroinvasion by VEE virus represent an important issue, particularly regarding immunoprophylaxis. The specific mechanism of neuroinvasion in the case of peripheral inoculation of virus is not completely understood, yet animal studies have elucidated some important features. In mice inoculated peripherally and subsequent to the development of viremia, virulent VEE virus is detectable in the brain, initially in the olfactory bulbs, and usually within 48 hours of infection.^{111,114,115} 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. Surgical or chemical ablation of the olfactory lining in mice reportedly delayed neuroinvasion via the olfactory nerves.¹¹⁴ An alternative theory, direct invasion of the brain across the blood-brain barrier,^{104,116} seems less compelling than the olfactory route.

The understanding of the mechanism of neuroinvasion after respiratory infection is more clear. An early and strong target of virulent VEE virus administered by aerosol has been shown to be the olfactory neuron.¹¹¹ 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 the development of viremia. In mice, both the nasal olfactory epithelium and the olfactory nerve axon

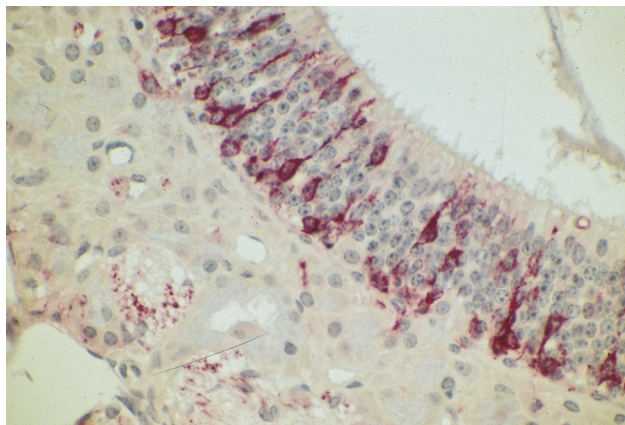


Fig. 12-4. Nasal tissue, BALB/c mouse, 2 days after exposure to aerosolized Venezuelan equine encephalitis (VEE) virus. Note immunoreactive olfactory epithelium and olfactory nerves. Alkaline phosphatase-labeled streptavidin method using rabbit antiserum to VEE virus (Mayer's hematoxylin counterstain, original magnification $\times 300$).

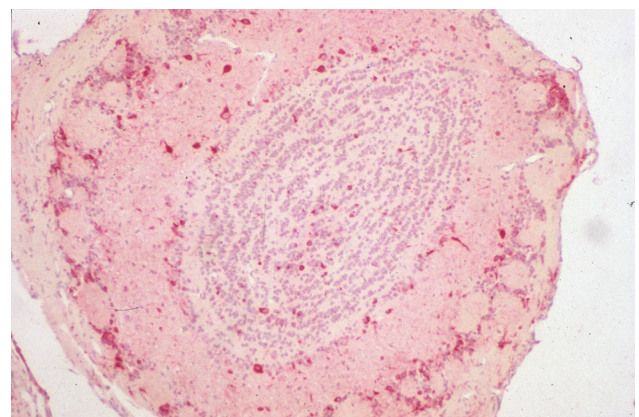


Fig. 12-5. Olfactory bulb, BALB/c mouse, 2 days after exposure to aerosolized Venezuelan equine encephalitis (VEE) virus. Note immunoreactive cells. Alkaline phosphatase-labeled streptavidin method using rabbit antiserum to VEE virus (Mayer's hematoxylin counterstain, original magnification $\times 150$).

bundles in the underlying connective tissue exhibit VEE virus antigen within 24 hours of aerosol infection (Figure 12-4), and the olfactory bulbs show viral infection shortly thereafter (Figure 12-5). In rhesus monkeys inoculated intranasally with VEE virus, the virus also 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.¹¹⁷ However, in inoculated monkeys whose olfactory nerves had been surgically removed, VEE virus 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. The teeth are another

early target of VEE administered peripherally or by aerosol,^{110,111,114} and the trigeminal nerves appear to carry VEE virus from the teeth into the brain as an alternate, although probably less significant, route of neuroinvasion. The mechanisms of neuroinvasion by peripheral versus aerosol administration are of significant practical concern because, as studies have shown, the immunological mechanisms of virus neutralization respective to each route can vary greatly.¹¹⁸⁻¹²⁰ The efficiency and rapidity of neuroinvasion after aerosol infection also place high demands on the vaccines used for immunoprophylaxis (vaccines are discussed later in this chapter). Neurons are the primary viral target in the brain and neuronal death by necrosis and/or apoptosis, accompanied by inflammatory changes, are the key consequences of infection.^{99-102,110,111}

CLINICAL DISEASE AND DIAGNOSIS

The three equine encephalomyelitis virus complexes within the *Alphavirus* genus, EEE, WEE, and VEE, 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 EEE and WEE viruses 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 IA, IB, and IC variants of VEE virus are pathogenic for equines and have the capacity for explosive epizootics with epidemic human disease. Epidemics of VEE affecting 20,000 to 30,000 people or more have been documented in Venezuela and Ecuador. In contrast to the other *Alphavirus* encephalitides (EEE and WEE), epizootic strains of VEE are mainly amplified in equines, rather than birds, so that equine disease normally occurs before reports of human disease. Enzootic VEE strains (variants ID, IE, and IF and subtypes II, III, IV, V, and VI) 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 12-2). 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 nonhuman primates, 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.¹²¹

After 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.¹²² 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 VEE virus are symptomatic,^{70,71} only a small percentage manifest neurological involvement.¹²³ 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.¹²⁴ 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.¹²⁵ However, for those who survive encephalitic involvement, neurological recovery is usually complete,¹²⁶ although one report documented motor disorders and an increased incidence of seizures in children after VEE outbreaks.¹²⁶ Abortions and increased fetal deaths have also been attributed to VEE virus infection.^{30,127} 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.^{99,128,129}

In the first 3 days of illness, leukopenia and elevated serum glutamic-oxaloacetic transaminase are common.

For those with CNS involvement, a lymphocytic pleocytosis of up to 500 cells per μl can be observed in the cerebrospinal fluid (CSF). The CSF pleocytosis may be acutely polymorphonuclear but soon becomes predominantly lymphocytic.

Specific diagnosis of VEE can be accomplished by virus isolation, serologic testing, or both.¹³⁰ During the first 1 to 3 days of symptoms of nonspecific febrile illness, VEE virus may be recovered from either the serum or the nasopharynx.¹³¹ Despite the theoretical possibility of person-to-person transmission of virus present in the nasopharynx, no such occurrences have been reported. Identification of the VEE subtype of an isolate involved can be accomplished by cross-neutralization tests. In nonhuman primates, 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.^{121,132} VEE virus can be isolated from the nasopharynx of nonhuman primates for up to 5 days after aerosol exposure of naïve animals. Hemagglutination inhibition, enzyme-linked immunosorbent assay (ELISA), or plaque-reduction neutralization antibodies appear as viremia diminishes. Complement-fixing antibodies make their appearance later during convalescence. VEE IgM antibodies are present in acute phase sera,⁷¹ and VEE IgM tests reportedly do not react with sera from patients with EEE or WEE.¹³³ Because patients with encephalitis typically come to evaluation later in the course of clinical illness, virus is recovered less often from them,¹³² and they usually have serum antibody by the time of clinical presentation.¹³⁴ Immunity after infection is probably lifelong to the homologous serotype, but cross-immunity may be weak or nonexistent to heterologous serotypes.⁵¹⁻⁵³ Thus, when viewed either as an endemic disease threat or as a potential biological warfare threat, adequate immunization will require polyvalent vaccines.

Eastern Equine Encephalitis

EEE is maintained in a natural transmission cycle between *Culiseta melanura* mosquitoes and passerine birds in swampy and forested areas. EEE outbreaks are typically recognized when severe equine or human encephalitis occurs near such areas.¹³⁵ During vectorborne EEE 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.^{136,137} However, the effect on morbidity and mortality of aerosol-acquired EEE infection (the expected route of infection in a biological warfare offensive) in humans is unknown, although animal studies indicate that EEE

by aerosol is lethal.¹³⁸ 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 disease¹³⁹; however, illness in children exhibits a more sudden onset.¹⁴⁰ In natural outbreaks, viremia occurs during the febrile prodrome,¹⁴¹ but is usually undetectable by the time clinical encephalitis develops, when hemagglutination inhibition and neutralizing antibodies become evident.¹⁴² 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.¹⁴³ During EEE outbreaks, the attack, morbidity, and fatality rates are highest in young children¹⁴⁴ and elderly persons.¹⁴⁵ Case fatality rates are estimated at 50% to 75%, but asymptomatic infections and milder clinical illness are underreported. The illness is characterized by rapid onset of high fever, vomiting, stiff neck, and drowsiness. Children frequently manifest generalized, facial, or periorbital edema. Motor involvement with paresis is common during the acute phase. Major disturbances of autonomic function, such as impaired respiratory regulation or excess salivation, may dominate the clinical picture. Between 30% and 70% of survivors have long-term neurological sequelae such as seizures, spastic paralysis, and cranial neuropathies. Cognitive impairment ranges from minimal brain dysfunction to severe dementia.

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. Specific diagnosis of EEE depends on virus isolation or serologic testing in which rising titers of hemagglutination inhibition, complement-fixing, or neutralizing antibodies are observed. IgM antibodies are usually detectable in acute-phase sera.¹³³ As with other alphaviruses, neutralization tests are the most specific. Immunohistochemistry can also be performed postmortem on fixed brain samples. In nonhuman primates exposed by aerosol to EEE, the period from fever onset until the animal is moribund is less than 48 hours regardless of dose.¹³⁸

Western Equine Encephalitis

Like VEE, WEE (by mosquito bite) is less virulent for adult humans than for equines and children, with lower rates of fatalities and neurological sequelae.¹⁴⁶

As with EEE, infants and elderly persons 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 infection. By aerosol, in nonhuman primates, the incubation period is 4 to 5 days.¹³⁸ 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.⁶⁴ However, the severity of the syndrome and the incidence of inapparent infection almost certainly depend on the strain and dose of virus, and the route of infection. Some unusual isolates show high virulence in laboratory animals,¹⁴⁷ and in one study of laboratory-acquired infections in adults, two of five patients died.¹⁴⁸ Symptoms usually begin with malaise, headache, and fever, followed by nausea and vomiting.¹⁴⁹ Telemetry data from nonhuman primates aerosol exposed to WEE found, in addition to fever, increases in heart rate and changes in electrocardiograph recordings, indicative of sinus tachycardia.^{150,151} 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 intensify, and somnolence or delirium may progress into coma. The severity of neurological involvement is inversely related to age, with over 90% of children younger than 1 year exhibiting focal or generalized seizures.¹⁵² Physical examination typically reveals nuchal rigidity, impaired sensorium, and upper motor neuron deficits with pathologically abnormal reflexes.

Patients with the most severe infections usually die within the first week of clinical illness, with overall 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.¹⁵³ 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.¹⁵⁴ In nonhuman primates, 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.¹⁵⁰

Viremia is rarely detectable by the time patients present with encephalitic symptoms, but IgM, hemagglutination inhibition, and neutralizing antibodies can generally be found by the end of the first week of illness, and they increase in titer during the next week.^{133,155,156} In nonhuman primates exposed to aerosolized WEE, the virus was not detectable in the serum or nasopharynx postexposure.¹⁵⁰ Low levels of virus were seen in spinal taps. Antibody response by ELISA or in-vitro neutralization was not detectable until day 9 postexposure, after animals had already died from the infection. Complement-fixing serologic responses generally appear in the second week and rise thereafter. Isolation of virus with up to a 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. WEE virus may occasionally be isolated from the CSF taken within the first 2 days of fever, and is frequently recovered from brain tissue on postmortem examination.¹⁵⁷ Natural infection presumably confers long-term immunity; however, it may not protect against aerosol exposure.¹⁵⁸

Differential Diagnosis of Alphavirus Encephalitis

Most acute infections with VEE and WEE 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 equines 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 *Brucella* species, *Yersinia pestis*, *Salmonella typhi*, *Coxiella burnetii*, and *Clostridium botulinum*. As with any diagnosis of meningoencephalitis, 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 clinical features are listed in Table 12-3. This list is not all-inclusive but suggests other viral encephalitides

TABLE 12-3
SOME IMPORTANT VIRAL CAUSES* OF
ENDEMIC ENCEPHALOMYELITIS

Virus Family	Genus	Species
<i>Togaviridae</i>	<i>Alphavirus</i>	Eastern equine Western equine Venezuelan equine
<i>Flaviviridae</i>		St. Louis Murray Valley West Nile Japanese Dengue Tick-borne complex
<i>Bunyaviridae</i>		LaCrosse Rift Valley Toscana
<i>Paramyxoviridae</i>	<i>Paramyxovirus</i> <i>Morbillivirus</i> <i>Henipavirus</i>	Mumps Measles Hendra Nipah
<i>Arenaviridae</i>	<i>Arenavirus</i>	Lymphocytic choriomeningitis Machupo Junin
<i>Picornaviridae</i>	<i>Enterovirus</i>	Poliovirus Coxsackievirus Echovirus
<i>Reoviridae</i>		Colorado tick fever
<i>Rhabdoviridae</i>	<i>Lyssavirus</i>	Australian bat lyssavirus Rabies
<i>Herpesviridae</i>	<i>Herpesvirus</i>	Herpes simplex virus types 1 and 2 Epstein-Barr virus Cytomegalovirus

*Not all-inclusive

that should be considered if a patient presents, *a priori*, with an encephalitic syndrome. Epidemiological, historical, and laboratory information are critical to differential diagnosis. Immediate and careful consideration must be given to treatable infections that may mimic viral encephalitis (Exhibit 12-1), 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 for-

ested areas during the summer. Encephalitic illness of equines in the surrounding locale is an important indication of ongoing transmission of encephalitic alphaviruses. Animal studies have indicated that 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 WEE. Examination of the CSF, including viral cultures, is critical in differentiating bacterial from viral infections, and infectious from noninfectious etiologies. Serum and CSF tests based on polymerase chain reaction techniques 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 specific therapy exists for the togaviral encephalitides; therefore, treatment is aimed at management of specific symptoms (eg, anticonvulsant medication and airway protection). The extremes of high fever occasionally produced by WEE infection in humans

EXHIBIT 12-1

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
- Carcinomatous meningitis

are a special problem among the arboviral encephalitides that may require aggressive antihyperthermia measures. The US Army has extensive experience with a live attenuated vaccine for VEE (TC-83) in humans. However, this vaccine is expected to protect efficiently against only IA/B and IC serotypes. The TC-83 vaccine is also reactogenic, with over 20% of vaccine recipients experiencing fever, malaise, and headache after the vaccination. Half of these patients experience symptoms severe enough to warrant bed rest for 1 to 2 days.

IMMUNOPROPHYLAXIS

Relevant Immune Effector Mechanisms

The equine encephalomyelitis viruses constitute both an endemic disease threat and a biological warfare threat; therefore, adequate immunoprophylaxis of military 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 are 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. Protection mediated by nonneutralizing antibodies to alphaviruses, directed mainly at epitopes on the E1 glycoprotein, has also been described.¹⁵⁹⁻¹⁶¹ In nonhuman primates and mice, protection from aerosol exposure correlated with serum neutralization or antibody titers.^{120,132,162,163}

Other nonspecific immune responses that occur following *Alphavirus* infection include the induction of interferon (IFN)¹⁶⁴⁻¹⁶⁷ and the activation of cytotoxic macrophages.¹⁶⁸ 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 VEE virus have shown that IFN α / β R knockout mice are more susceptible to infection.¹⁶⁹⁻¹⁷¹ Pre- and postexposure administration of IFN or inducers of IFN in vivo may be effective for protection against alphaviruses.^{172,173} 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 the TrD strain of VEE virus and were partially protected

Use of an effective vaccine in horses would prevent outbreaks of epizootic VEE, because equines are the major amplifying species for VEE virus. Vaccination of horses is not a useful public health tool for EEE, WEE, or enzootic VEE, however, because horses are not important as amplifying hosts for these diseases. Investigational formalin-inactivated vaccines for humans are available for WEE and EEE, but they require multiple injections and are poorly immunogenic. Integrated mosquito control measures also have significant impact on ameliorating epidemic transmission.

from inhalation challenge when administered pegylated IFN- α on days -2 and +5.¹⁷⁴ Pretreating mice with poly IC afforded partial protection against peripheral challenge with EEE virus,¹⁶⁹ and poly-ICLC similarly induces protection against respiratory challenge with WEE virus.¹⁷³ Although these studies clearly indicate the importance of IFNs in host resistance to *Alphavirus* infections, further study is necessary to determine the efficacy of IFN- α for prophylactic or therapeutic use in humans. There have also been reports of virus-specific cytotoxic T-cell responses induced against alphaviruses,¹⁷⁵⁻¹⁷⁸ although it has proven difficult to show that these T-cell responses play a significant role in protection.

Passive Immunization

Passive transfer of neutralizing antisera or monoclonal antibodies to naive recipients protects animals from subsequent parenteral challenge with homologous VEE strains.^{160,167,179} Passive transfer of nonneutralizing, anti-E1 monoclonal antibodies directed against appropriate epitopes is also protective against Sindbis,¹⁵⁹ WEE,¹⁶¹ and VEE¹⁶⁰ viruses. However, for the respiratory route of infection, uniform protection was not observed after passive transfer of hyperimmune serum to hamsters¹⁶¹ or neutralizing monoclonal antibodies to mice,¹⁸⁰ 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 WEE virus was observed if immune serum was given no more than 3 days before virus exposure.^{181,182} 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.¹⁸³ 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.^{181,182,184-186} Other researchers have suggested that postinfection serum transfer may also cause a more severe pathology, or may merely delay the onset of disease symptoms.¹⁸⁷ Aggressive serotherapy following infections of two laboratory workers who developed acute WEE encephalitis resulted in the survival of one patient¹⁸⁸ but was ineffective in the second patient.¹⁸³

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.¹⁸⁹ 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, indicates that serotherapy would be an ineffective means of treatment for these virus infections, unless initiated early in the course of disease.

Active Immunization

Vaccines available for use against the equine encephalomyelitis viruses include TC-83, which is a live attenuated vaccine for VEE, and inactivated vaccines for VEE, EEE, and WEE. All these vaccines are used under the Food and Drug Administration's investigational new drug status. The characteristics of these vaccines and the responses induced in human vaccinees are summarized in Table 12-4.

Live Vaccines

The TC-83 VEE vaccine, which was developed in 1961 by serial passage of the virulent TrD strain in fetal guinea pig heart cells,¹⁹⁰ is administered subcutaneously (0.5 mL) at 1×10^4 to 2×10^4 plaque-forming units per dose. The vaccine was used initially in laboratory and field personnel at risk for exposure to VEE,¹⁹¹ and over 6,000 people received the vaccine between 1964 and 1972.¹⁹¹ For reasons that remain unclear, approximately 20% of the people who receive TC-83 fail to make a minimum neutralizing antibody response and probably would not be protected should they be exposed to the virus. Another 25% of vaccine recipients experience clinical reactions ranging from mild transient symptoms to fever, chills, sore throat,

TABLE 12-4
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

and malaise sufficient to require bed rest.¹⁹² 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.¹⁹³ In humans, documented vaccine-breakthrough infections have been attributed largely to exposure to heterologous, enzootic strains of VEE virus.⁵¹⁻⁵³ Although pregnant mares were not adversely affected by TC-83,¹⁹⁴ pregnant women are advised not to receive the TC-83 vaccine, because wild-type VEE may have been associated with spontaneous abortions or stillbirths during an epidemic in Venezuela in 1962.¹⁰⁰

In animals, TC-83 vaccination will protect hamsters from a lethal VEE subcutaneous or aerosol challenge,¹⁶² although up to 20% of hamsters may die of vaccine reactions.^{106,195} Subcutaneous vaccination of monkeys¹¹² with the vaccine produces neutralizing antibody responses in serum and protection from virulent VEE virus delivered by peripheral or intranasal challenge. However, TC-83 provides only partial protection against aerosol challenge in outbred mice.¹¹⁶ TC-83 has been extensively administered to horses, burros, and mules, in part because large numbers of equines were vaccinated during the 1969–1970 epizootic. TC-83 vaccination produces febrile responses and leukopenia in some equines,^{196,197} but neutralizing antibody responses to homologous (serotype IA) virus eventually develop in 90% of these animals.^{196,198} Although it was difficult to accurately assess vaccine efficacy under the conditions of an ongoing epizootic, herds of animals known to have been vaccinated at least 2 weeks before any disease occurrence in the area did not sustain any VEE-related deaths, whereas unimmunized herds experienced up to 60% mortality rates.¹⁹²

The phenomenon of vaccine interference, in which prior immunity to heterologous alphaviruses inhibits vaccine viral 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,^{199,200} 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 vaccination with a second, different *Alphavirus* vaccine.²⁰¹

Inactivated Vaccines

Against VEE (C-84). Early attempts to develop an inactivated vaccine against VEE resulted in preparations that contained residual live virus and caused disease in 4% of those who received it.^{184,202} Develop-

ment of an inactivated VEE vaccine (C-84) was begun, using the TC-83 attenuated strain of virus, because of the problems associated with incomplete inactivation.²⁰³ Initial clinical trials with the C-84 inactivated vaccine were begun in 1976 in 14 volunteers previously vaccinated with TC-83, and subsequently in 14 naive volunteers.²⁰⁴ 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 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.¹⁹¹ However, the observation that hamsters given C-84 vaccine were protected from subcutaneous challenge but not from an aerosol exposure to VEE virus¹⁶² 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.

Against EEE and WEE. The PE-6 strain of EEE virus was passed in primary chick-embryo cell cultures, and then it was formalin-treated and lyophilized to produce an inactivated vaccine for EEE.²⁰⁵ 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 neutralizing antibody titers. In initial clinical trials, mild reactions to the vaccine were observed, and immunogenicity was demonstrated.²⁰⁶ The vaccine was given to 896 at-risk laboratory workers between 1976 and 1991. No significant clinical reactions were 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.¹⁹¹

The WEE vaccine was similarly prepared using the B-11 or CM-4884 virus strain, and it caused only mild clinical reactions when administered to WEE-naive individuals.²⁰⁷ Between 1976 and 1990, 359 laboratory workers were vaccinated with the WEE vaccine. Long-term follow-up studies have indicated that administration of three doses of 0.5 mL 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 doses.²⁰⁷

Active programs are ongoing in a variety of government and university laboratories to develop safe and effective vaccine alternatives.

SUMMARY

The equine encephalomyelitis viruses consist of three antigenically related viruses within the *Alpha-virus* genus of the family *Togaviridae*: (1) VEE, (2) WEE, and (3) EEE. These viruses, which are vectored in nature by various species of mosquitoes, 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 after infection of humans differs. EEE is the most severe of the arbovirus encephalitides, with case fatality rates of 50% to 70%. WEE virus 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 after VEE virus infection, but essentially all infected individuals develop a pro-

trating 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, which can be produced in large amounts in inexpensive and unsophisticated systems, are relatively stable and readily amenable to genetic manipulation. For these reasons, the equine encephalomyelitis viruses are considered classic biological warfare threats. No specific therapy exists for infections caused by these viruses. A live attenuated vaccine for VEE (TC-83) and inactivated vaccines for VEE, EEE, and WEE have been developed and are used under the Food and Drug Administration's investigational new drug status. Although these vaccines are useful in protecting at-risk individuals, they have certain disadvantages, and improved vaccines are under development.

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Chapter 13

VIRAL HEMORRHAGIC FEVERS

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INTRODUCTION

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INTRODUCTION

Viral hemorrhagic fever (VHF) is an acute febrile syndrome characterized by systemic involvement, which includes generalized bleeding in severe infections. Patients with VHF manifest combinations of malaise, prostration, generalized signs of increased vascular permeability, and coagulation abnormalities. Although the more severely ill patients manifest bleeding, this does not result in a life-threatening loss of blood volume. To a certain extent, however, it indicates damage to the vascular endothelium and is an index of disease severity in specific target organs. Much of the disease appears to be caused by dysregulation of the innate immune response, although replication of these hemorrhagic fever (HF) viruses in target cells and tissues can directly contribute to the pathological manifestations of VHF. Factors that may contribute to this subversion of the host immune response include the rapid infection and impairment of dendritic cells, a sudden and enigmatic death of lymphocytes, and the release of a variety of mediators from virus-infected cells that subsequently alter vascular function and trigger the coagulation disorders that epitomize these infections.

The viral agents causing severe HF, which are taxonomically diverse, are all single-stranded RNA viruses that can infect humans through contact with contaminated animal reservoirs or arthropod vectors. Under natural conditions, these viruses cause significant infectious diseases, although their geographical ranges may be tightly circumscribed. The relatively recent advent of jet travel coupled with human demographics increase the opportunity for humans to contract these infections; from time to time, sporadic cases of VHF are exported from endemic areas to new

areas. Clinical and epidemiological data on VHFs are sparse; outbreaks are sporadic and unexpected, and typically develop in geographical areas where cultural customs and logistical barriers encumber systematic investigations.

Because many VHFs spread easily in hospitals to patients and staff alike, causing high morbidity and mortality, they gained public notoriety in the past decade from the enormous interest and fear generated by the news media. Ebola, an HF virus with a high case-fatality rate (near 90% in some outbreaks), dramatic clinical presentation, and lack of effective specific treatment, was highly publicized when a new Ebola species was isolated in a suburb of Washington, DC, in 1989.¹ Progress in understanding the genesis of the pathophysiological changes that make Ebola and other HF viral infections of humans so devastating has been slow, primarily because special containment is required to safely work with most of these viruses.

Many of the VHF agents are highly infectious by aerosol. Most VHF agents are also stable as respirable aerosols, which means that they satisfy at least one criterion for weaponization, and some have potential as biological terrorism and warfare threats. Most of these agents replicate in cell culture to concentrations sufficiently high to create a small terrorist weapon, one suitable for introducing lethal doses of virus into the air intake of an airplane or office building. Some replicate to higher concentrations, with obvious potential ramifications. Because the VHF agents cause serious diseases with high morbidity and mortality, their existence as endemic disease threats and as potential biological warfare weapons suggests a formidable potential impact on public health.

HISTORY AND EPIDEMIOLOGY

Natural Disease

Under natural conditions members of the *Arenaviridae*, *Bunyaviridae*, *Filoviridae*, and *Flaviviridae* (Table 13-1) that cause VHF have specific geographic distribution and diverse modes of transmission. Although the natural reservoir for *Filoviridae* remains unknown, as a group, the HF viruses are linked to the ecology of their vectors or reservoirs, whether rodents or arthropods. These characteristics have great significance not only in the natural transmission cycle for arenaviruses and bunyaviruses (rodents to humans) and for flaviviruses (arthropods), but also in the potential for nosocomial transmission. Most reservoirs tend to be rural, and

a patient's history of being in a rural locale is an important factor to consider when reaching a diagnosis. Human-to-human spread is possible for most of the HF viruses. The majority of person-to-person spread has been attributed to direct contact with infected blood and body fluids. Airborne transmission of VHF agents appears to be an infrequent event, but cannot categorically be excluded as a mode of transmission.

Arenaviridae

The name *arena* is derived from the Latin words "arenosus" (sandy) and "arena" (sand) in recognition of the sand-like ribosomal contents of virions in

TABLE 13-1
VIRAL HEMORRHAGIC FEVERS OF HUMANS

Virus Family Genus	Virus	Disease	Natural Distribution	Source	Incubation (Days)
Arenaviridae					
<i>Arenavirus</i>	Lassa	Lassa fever	West Africa	Rodent	5–16
	Junin	Argentine HF	South America	Rodent	7–14
	Machupo	Bolivian HF	South America	Rodent	9–15
	Sabia	Brazilian HF	South America	Rodent	7–14
	Guanarito	Venezuelan HF	South America	Rodent	7–14
	Whitewater Arroyo	Unnamed HF	North America	Rodent	Unknown
Bunyaviridae					
<i>Nairovirus</i>	Crimean-Congo HF	Crimean-Congo HF	Africa, Central Asia, Eastern Europe, Middle East	Tick	3–12
	<i>Phlebovirus</i>	Rift Valley fever	Rift Valley fever	Mosquito	2–6
	<i>Hantavirus</i>	Agents of HFRS	HFRS	Rodent	9–35
Filoviridae					
<i>Ebolavirus</i> [†]	Ebola	Ebola HF	Africa	Unknown	2–21
	<i>Marburgvirus</i>	Marburg HF	Africa	Unknown	2–14
Flaviviridae					
<i>Flavivirus</i>	Dengue	Dengue HF	Asia, Africa, Pacific, Americas	Mosquito	Unknown
	Yellow fever	Yellow fever	Africa, tropical Americas	Mosquito	3–6
	Omsk HF	Omsk HF	Central Asia	Tick	2–9
	Kyasanur forest disease	Kyasanur forest disease	India	Tick	2–9

HF: hemorrhagic fever; HFRS: hemorrhagic fever with renal syndrome

*The agents of hantavirus pulmonary syndrome were isolated in North America.

[†]There are four species of Ebola: Zaire, Sudan, Reston, and Ivory Coast.

thin section under the electron microscope. The family *Arenaviridae* contains a single genus, *Arenavirus*. However, the arenaviruses are divided into an Old World group (eg, Lassa virus) and a New World group (South American and North American HF viruses) by phylogenetic analysis of RNA and serology. The New World complex is further divided into three major clades: A, B, and C. All of the viruses causing HF belong to clade B.² Arenaviruses survive in nature by a lifelong association with specific rodent reservoirs. Rodents spread the virus to humans, and outbreaks can usually be related to some perturbation in the ecosystem that brings humans in contact with rodents or material contaminated by rodent products. Arenaviruses initiate infection in the nasopharyngeal mucosa.

Lassa fever made a dramatic appearance in 1969 when an American nurse working at a modest mission station in Lassa, a small town in northeastern

Nigeria, became ill and started a chain of nosocomial infections that extended from healthcare workers in Africa to laboratory workers in the United States. Lassa virus produces Lassa fever, a major febrile disease of West Africa that causes 10% to 15% of adult febrile admissions to the hospital and perhaps 40% of nonsurgical deaths.³ Lassa virus infects 100,000 to 300,000 people annually in West Africa, kills 5,000 to 10,000, and leaves approximately 30,000 deaf.^{3,4} Lassa fever causes high mortality in pregnant women and is also a pediatric disease. Most Lassa virus infections are traceable to contact with the carrier rodent, the rat (*Mastomys natalensis*), but nosocomial transmission is also possible. Lassa fever has periodically been imported to Europe, the United States, Canada, and Japan by travelers from West Africa.⁵ Since 2000 at least five fatal Lassa fever cases have occurred in the United Kingdom, Germany, the Netherlands, and the United States.^{6,7}

Argentine HF (AHF) was described in 1943, and Junin virus was first isolated from one of its victims in 1958. Junin virus, which is carried by field voles such as *Calomys musculinus* and *Calomys laucha*, is primarily associated with agricultural activities in the pampas of Argentina, where there have been 300 to 600 cases per year since 1955.⁸ Transmission is airborne from fomites, contaminated food or water, or abrasions to the skin. Direct person-to-person transmission is rare.

In 1959 physicians at the Beni department of Bolivia noted a sporadic hemorrhagic illness in patients from rural areas, which soon became known as Bolivian HF. In 1963 Machupo virus was isolated from patients with Bolivian HF, and shortly thereafter voles (*Calomys callosus*) were identified as the rodent reservoir.⁹ Machupo virus produced several outbreaks of disease in the 1960s, but more recently Bolivian HF has manifested only sporadically; there was a cluster of cases in 1994. Transmission is through contaminated food and water and direct contact through breaks in the skin; there is only rare documentation of human-to-human transmission.

In 1989 an outbreak of VHF involving several hundred patients in the municipality of Guaranito, Portuguesa state, Venezuela, led to the isolation of Guaranito virus and identification of its probable animal reservoir, the cotton rat (*Sigmodon hispidus*).¹⁰ Sabia virus caused a fatal VHF infection in Brazil in 1990,¹¹ a severe laboratory infection in Brazil in 1992, and another laboratory-acquired infection in the United States in 1994. The most recently recognized arenavirus linked to VHF is Whitewater Arroyo virus, which apparently caused three fatal cases of HF in California between 1999 and 2000.¹²

Bunyaviridae

Of the five genera that comprise the family *Bunyaviridae*, three genera contain viruses that cause HF: (1) *Phlebovirus* (eg, Rift Valley fever virus); (2) *Nairovirus* (eg, Crimean-Congo HF virus); and (3) *Hantavirus* (eg, Hantaan virus). *Bunyaviridae* is transmitted by arthropods (primarily mosquitoes, ticks, and phlebotomine flies), or, as is the case for hantaviruses, by contact with rodents or rodent products. Transmission by aerosol is also documented.

The phlebovirus Rift Valley fever (RVF) virus, which causes RVF, is a significant human pathogen. Outbreaks of this major African disease often reflect unusual increases in mosquito populations.¹³ RVF virus, which primarily affects domestic livestock, can cause epizootic disease in domestic animals. RVF was first described in 1931 as an enzootic hepatitis among sheep, cattle, and humans in Kenya.¹⁴ During

1950–1951, an epizootic of RVF in Kenya resulted in the death of about 100,000 sheep. An RVF epizootic can lead to an epidemic among humans who are exposed to diseased animals. Risk factors for human infection include contact with infected blood, especially in slaughterhouses, and handling of contaminated meat during food preparation. Exposure to aerosols of RVF virus is a potential source of infection for laboratory workers. In 2000 RVF spread for the first time beyond the African continent to Saudi Arabia and Yemen, affecting both livestock and humans.¹⁵

Crimean-Congo HF (CCHF) is a zoonotic disease transmitted not only through the bite of at least 29 species of ticks, of which *Hyalomma marginatum* is the most important, but also by exposure to infected animals or their carcasses, contact with blood and bodily secretions of infected persons, and by aerosol. The agent of CCHF is a *Nairovirus*. Although descriptions of this illness can be traced to antiquity, this disease was first recognized in 1944–1945 when a large outbreak occurred in the Steppe region of western Crimea among Soviet troops and peasants helping with the harvest. In 1956 a similar illness was identified in a febrile child from what was then the Belgian Congo (now the Democratic Republic of the Congo), but it was not until 1969 that researchers realized that the pathogen causing Crimean HF was the same as that responsible for the illness in the Congo. The linkage of the two place names resulted in the current name for the disease and the virus. CCHF is endemic in many countries in Africa, Europe, and Asia; it causes sporadic, yet particularly severe, VHF in endemic areas.¹⁶ CCHF is often associated with small, hospital-centered outbreaks, owing to the profuse hemorrhage and highly infective nature of this virus in humans exposed by aerosol. An HF outbreak on the Pakistani-Afghan border during the 2001–2002 US campaign against terrorists is suspected to have been caused by the CCHF virus, and various media outlets have reported that CCHF was confirmed by a laboratory in South Africa.

Hantaviruses, unlike other bunyaviruses, are not transmitted by infected arthropods; rather, contact with infected rodents and their excreta leads to most human infections. However, person-to-person transmission was described during a recent outbreak of hantavirus pulmonary syndrome (HPS) in southwest Argentina,¹⁷ and researchers have also documented transmission by aerosol.¹⁸ Of the more than 20 known types of hantaviruses, at least nine (Hantaan, Seoul, Puumala, Dobrava, Sin Nombre, New York, Black Creek Canal, Andes, and Bayou) hantaviruses can cause significant clinical illness. Each virus has its own rodent vector, geographic distribution, and clinical expression. The poor sanitary conditions of combat promote exposure to rodents. A

review of illness during the US Civil War, World War I, and World War II suggests that outbreaks of hantaviral infections occurred among troops. Hantaviral disease was described in Manchuria along the Amur River, and later among United Nations troops during the Korean War, where it became known as Korean HF.¹⁹ The prototype virus from this group, Hantaan, which causes Korean HF with renal syndrome (HFRS), was isolated in 1977. The reservoir host for Hantaan virus is the striped field mouse (*Apodemus agrarius*).

Hantaan virus is still active in Korea, Japan, and China. Seoul virus, which is carried mainly by the house rat (*Rattus norvegicus*), causes a milder form of HFRS and may be distributed worldwide. Other hantaviruses associated with HFRS include the Puumala virus, which is associated with bank voles (*Clethrionomys glareolus*). An epidemic in 1993 in the Four Corners region of the United States led to the identification of a new hantavirus (Sin Nombre virus), and eventually to identification of several related viruses (Black Creek Canal, New York, Andes, and Bayou); all of these have been associated with HPS.^{20,21} The classical features of the syndrome of acute febrile illness associated with prominent cardiopulmonary compromise have been extended to clinical variants, including disease with frank hemorrhage.²¹

Filoviridae

Marburg virus and Ebola virus, the causative agents of Marburg and Ebola HF, respectively, represent the two genera that comprise the family *Filoviridae*. The *Marburgvirus* genus contains a single species: *Lake Victoria marburgvirus*. The *Ebolavirus* genus is divided into four distinct species: (1) *Ivory Coast ebolavirus*, (2) *Reston ebolavirus*, (3) *Sudan ebolavirus*, and (4) *Zaire ebolavirus*. By electron microscopy, filoviruses have a highly unusual filamentous appearance. The term filovirus was derived from "filo," which is Latin for thread. Marburg virus was first recognized in 1967 when three simultaneous outbreaks of a lethal VHF epidemic occurred at Marburg and Frankfurt, Germany, and Belgrade, Yugoslavia, among laboratory workers exposed to the blood and tissues of African green monkeys (*Chlorocebus aethiops*) imported from Uganda. Secondary transmission to medical personnel and family members was also documented.²² A clinician recognized the initial outbreak in Marburg.²² Thirty-one patients became infected, and seven died. The 23% human mortality and bizarre morphology of the newly discovered virus had a great psychological impact and led to new quarantine procedures for imported animals. During the next two decades, Marburg virus was associated with sporadic, isolated, usually

fatal cases among residents and travelers in southeast Africa. In 1998–2000, an outbreak of Marburg HF in Durba, Democratic Republic of the Congo, was linked to individuals working in a gold mine.²³ In 2004–2005 there was a Marburg virus outbreak in Angola that caused over 200 deaths (90% mortality).²⁴

Ebola viruses, taxonomically related to Marburg viruses, were first recognized during near-simultaneous explosive outbreaks in 1976 in small communities in the former Zaire (now the Democratic Republic of the Congo)²⁵ and Sudan.²⁶ Reuse of unsterilized needles and syringes and nosocomial contacts caused significant secondary transmission. These independent outbreaks involved serologically distinct viral species. The Ebola-Zaire outbreak involved 318 cases and 280 deaths (88% mortality), and the Ebola-Sudan outbreak involved 280 cases and 148 deaths (53% mortality). Since 1976 Ebola virus has appeared sporadically in Africa, causing several small- to mid-size outbreaks between 1976 and 1979. In 1995 a large epidemic of Ebola-Zaire HF involving 315 cases occurred, with an 81% case fatality rate, in Kikwit, a community in the former Zaire.²⁷ Meanwhile, between 1994 and 1996, the Ebola-Zaire virus caused smaller outbreaks in Gabon.²⁸ In 2000 Gulu, Uganda, suffered a large epidemic of VHF attributed to the Sudan species of Ebola virus.²⁹ More recently, Gabon and the Republic of Congo suffered small VHF outbreaks attributed to Ebola-Zaire virus. The most recent outbreaks in Gabon and the Republic of Congo also involved a catastrophic decline in populations of great apes, which may have a role in transmission to humans.^{30,31}

In 1989 a third species of Ebola virus appeared in Reston, Virginia, in association with an outbreak of VHF among cynomolgus monkeys (*Macaca fascicularis*) imported to the United States from the Philippine Islands.¹ Hundreds of monkeys were infected (with high mortality) in this episode, but no human cases occurred, although four animal caretakers seroconverted without overt disease. Epizootics in cynomolgus monkeys recurred at other facilities in the United States and Europe through 1992 and again in 1996. The lack of human disease in these episodes suggests that the Reston species of Ebola may be less pathogenic to humans, although the pathogenic potential in humans is unknown. A fourth species of Ebola virus, Ivory Coast, was identified in Côte d'Ivoire in 1994; this species was associated with chimpanzees, and only one nonfatal human infection was identified.³²

Little is known about the natural history of filoviruses. Surveys in Central Africa of a variety of species of animals and arthropods have yet to conclusively identify a reservoir host. Laboratory studies have shown that fruit and insectivorous bats can support

replication and circulation of high titers of Ebola virus without showing overt illness, suggesting that they could serve some role in the natural history of filoviruses.³³ Recently, an ecological study in Gabon and the Republic of the Congo showed asymptomatic infection by Ebola-Zaire virus in three species of fruit bats; however, no isolate was recovered from any of these bats.³⁴

Flaviviridae

Viruses responsible for HF of the family *Flaviviridae* (type species yellow fever virus) are members of the genus *Flavivirus*, including yellow fever, dengue, Kyasanur forest disease, and Omsk. Mosquitoes transmit yellow fever, found throughout Africa and South America, and dengue, found throughout the Americas, Asia, and Africa.³⁵ Yellow fever was likely transported from Africa to the Americas during the slave trade. Yellow fever accounts in the Americas date to a probable 1648 outbreak in the Yucatan Peninsula. Carlos Finlay, a Cuban physician, identified *Aedes aegypti* as a likely vector and promulgated the theory of mosquito transmission. Dr Finlay supplied the Walter Reed commission with mosquito eggs and facilitated the US experiments that demonstrated that an extrinsic incubation period in the mosquito was needed before transmission. Benjamin Rush described classic dengue as “breakbone fever” in 1789. In 1954 dengue HF/dengue shock syndrome (DHF/DSS) was described in the Philippines, and became known as Philippine HF. There are four dengue virus serotypes: 1, 2, 3, and 4. DHF/DSS manifests in infants born to dengue-immune mothers, and in persons older than 1 year with prior immunity to one serotype of dengue virus who became infected with another serotype. Humans are the reservoir of dengue virus, but a jungle cycle involving forest mosquitoes and monkeys, similar to that associated with yellow fever, is recognized. In 1981 Cuba reported the first serologically confirmed case of DHF/DSS outside of Asia. Both yellow fever and dengue have had major impact on military campaigns and military medicine.

The tick-borne flaviviruses include the agents of Kyasanur forest disease of India³⁶ and Omsk HF found mainly in regions of Siberia.³⁷ Kyasanur forest disease, also called “monkey disease,” was first described in 1957 in the Kyasanur forest of Mysore, India. Both diseases have a biphasic course; the initial phase includes a prominent pulmonary component, followed by a neurological phase with central nervous system manifestations. Both diseases can also manifest as HF. Alkhurma virus was isolated in 1995 from patients with HF in Saudi Arabia³⁸ and appears to be closely

related to Kyasanur forest disease. Evidence suggests that transmission to humans can occur either by contamination of a skin wound with the blood of an infected vertebrate or bites of an infected tick, or by ingestion of unpasteurized contaminated milk.

Potential Role in Biological Warfare and Terrorism

Public concern about the dangers posed by VHF reflects their potential for high morbidity and mortality, their potential spread from increased international commerce and air travel, and the heightened bioterrorism awareness advanced by the events surrounding September 11, 2001. The Centers for Disease Control and Prevention has classified most of the viruses causing HF as category A bioweapon agents.³⁹ This classification identifies agents associated with high mortality rates, ease of dissemination or person-to-person transmission, and potential for major public panic and social disruption, and that require special action for public health preparedness.

The Japanese studied VHF for use in warfare during their activities with Unit 731; specifically, they studied hantaviruses and noted that rodents served as reservoirs.⁴⁰ The Soviet Union, Russia, and the United States weaponized several HF viruses⁴¹⁻⁴³ and both the Soviet Union and Russia produced large quantities of Ebola, Marburg, Lassa, Junin, and Machupo viruses until 1992.^{41,43} Soviet researchers determined that only a few virions of Marburg virus administered aerogenically can produce a lethal infection in monkeys,⁴⁴ and they showed that small doses of Ebola virus produced lethal infection in monkeys when administered by aerosol.⁴⁵ Many studies revealed that aerosol preparations of Ebola,^{45,46} Marburg,^{44,47} Lassa,⁴⁸ and Junin⁴⁹ viruses could produce lethal infection of nonhuman primates. Some argue that these viruses are too dangerous to develop as weapons because no effective vaccines or therapies exist; however, the Japanese cult Aum Shinrikyo’s attempt in 1992 to obtain Ebola virus as part of a covert effort to develop biological weapons contradicts this view.⁵⁰

Evidence suggests that North Korea weaponized yellow fever virus.^{42,51} Moreover, the US offensive biological weapons program developed yellow fever and RVF viruses as weapons before terminating its program in 1969.⁴² In 1970 the World Health Organization projected that an aerosol attack with 50 kg of RVF virus on a municipality of 500,000 residents would reach an estimated downwind distance of 1 km and cause 35,000 casualties, with a mortality rate of 0.5%.⁵² Use of HF viruses with higher mortality rates such as Ebola virus or Marburg virus would ostensibly cause more significant morbidity and mortality.

The Working Group on Civilian Biodefense recently excluded the viruses causing dengue HF, CCHF, and the agents of HFRS as potential biological weapons.⁵³ The group excluded dengue virus because it is not transmissible by small-particle aerosol.⁵⁴ Exclusion of

CCHF and the agents of HFRS as tools of bioterrorism is based primarily on technical problems; most importantly, these agents do not readily grow to high concentrations in cell culture, which is necessary for weaponization of an infectious organism.⁵³

AGENT CHARACTERISTICS

Despite the diversity of the four families of viruses (*Arenaviridae*, *Bunyaviridae*, *Filoviridae*, and *Flaviviridae*) that contribute pathogens to the group of VHF agents, these viruses share common characteristics. The viruses causing severe HF have a single-stranded RNA genome and a lipid envelope, making them susceptible to detergents, as well as to low pH environments and household bleach. Conversely, they are stable at neutral pH, especially when protein is present. These viruses also are stable in blood for long periods and can be isolated from a patient's blood after weeks of storage at refrigerator or ambient temperatures. For example, Ebola virus was successfully cultured from dried blood found in syringes that had been stored at room temperature for about a month during a Central African outbreak in 1995.⁵⁵ Other examples include a study showing that yellow fever virus blotted onto filter paper discs, air dried, and stored at room temperature could be successfully cultured 90 days later.⁵⁶

All HF viruses are biosafety level 3 or biosafety level 4 agents, except for the dengue viruses, because these viruses tend to be stable and highly infectious as fine-particle aerosols and produce disease with high morbidity and mortality. The HF viruses vary considerably in morphology from typical small isometric or moderately sized spherical virions to highly unusual pleomorphic or filamentous particles (Figure 13-1).

Arenaviridae

Arenavirus particles contain a genome consisting of two ambisense single-stranded RNA molecules, designated S (small) and L (large), of about 3.4 kb and 7.2 kb in length, respectively.⁵⁷ The S segment contains two genes that encode three structural proteins: the nucleoprotein (NP or N), and the envelope glycoproteins (GP1 and GP2). The L segment contains two genes that encode two proteins: the viral polymerase (L protein) and the Z protein. NP and L associate with the genomic RNA in a ribonucleoprotein complex or nucleocapsid structure. Z protein functions as a matrix protein and is responsible for the formation of viral particles.⁵⁸ GP1 and GP2 are initially synthesized as a precursor molecule, GPC, which is posttranslationally cleaved.⁵⁹ GP2 homotetramers bind by ionic interactions with GP1 homotetramers, which make up the globular head of the

glycoprotein spikes.⁶⁰ GP1 is the portion of the surface glycoprotein spike that is the effector for receptor binding,^{61,62} whereas the GP2 is the viral fusion protein.^{63,64}

Bunyaviridae

Bunyavirus particles contain three single-stranded RNA genome segments designated large (L), medium (M), and small (S), which vary in size among the genera.

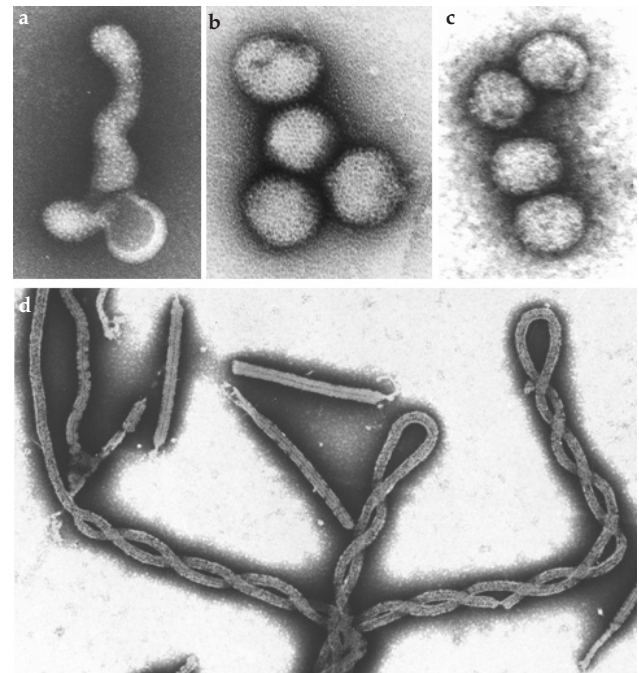


Fig. 13-1. Transmission electron micrographs of negatively stained hemorrhagic fever viral particles. (a) Junin virus. Arenavirus particles range in morphology from highly pleomorphic as shown in this field to mainly spherical. Virion sizes range from 50 to 300 nm with a mean of 100 to 130 nm. (b) Rift Valley fever virus. Bunyaviral particles are roughly spherical and range in diameter from 90 to 120 nm. (c) Yellow fever virus. Flaviviral particles are essentially isometric and consistent in size, ranging from 40 to 50 nm in diameter. (d) Ebola virus. Filoviral particles are mostly filamentous and vary in length up to 14,000 nm with a uniform diameter of 80 nm. Mean unit length is about 1,000 nm. Other forms of filoviral particles include U-shaped, "6"-shaped, or circular configurations; branching of filamentous particles can also occur.

The L segment encodes an RNA-dependent RNA polymerase (L), the M segment encodes two virion glycoproteins (G1 and G2) and in some viruses a non-structural protein (NSm), and the S segment encodes a nucleoprotein (N) and in some viruses a nonstructural protein (NSs).⁶⁵⁻⁶⁷ The structural proteins (L, N, G1, G2) are encoded in viral cRNA. NSs are encoded in the M segment cRNA and the S segment vRNA of phleboviruses. Hantaviruses and nairoviruses use negative-sense coding strategies, whereas phleboviruses use ambisense coding strategies. The functions of the NSs have not been fully delineated; NSs protein may control the activity of the viral polymerase and was proposed to block interferon (IFN) production.⁶⁸

Filoviridae

Ebola and Marburg virus particles contain an approximately 19-kb, single, negative-stranded, linear RNA genome that is noninfectious. The genome encodes seven structural proteins with the following gene order: 3' leader, nucleoprotein (NP), virion protein (VP) 35, VP40, glycoprotein (GP), VP30, VP24, polymerase L protein, and 5' trailer.^{69,70} Four of these proteins, NP, VP30, VP35, and L, associate with the genomic RNA in a ribonucleoprotein complex, whereas the three remaining proteins, GP, VP24, and VP40, are associated with the membrane. GP is the surface glycoprotein that forms the spikes on the virion and is the effector for receptor binding and membrane fusion.^{71,72} GP is synthesized as a precursor molecule, GP₀, which is postrationally cleaved by furin or a furin-like endoprotease into two subunits, GP₁ and GP₂; these subunits are linked by disulfide bonding to form a heterodimer.^{73,74} Homotrimers of GP₁-GP₂ comprise the virion spikes. The unique organization of the GP gene of Ebola virus provides an important distinction between Marburg and Ebola viruses. The Marburg virus GP gene encodes a single product, the GP, in a conventional open reading frame, whereas all of the Ebola viruses encode the GP in two open reading frames that are expressed through transcriptional editing.^{75,76} The primary gene product of the Ebola GP gene is not the GP, but rather a smaller, nonstructural, secreted glycoprotein (sGP), which is efficiently secreted

from infected cells. VP40 functions as a matrix protein and is responsible for the formation of the filamentous particles.⁷⁷ VP24 is a minor viral protein whose functions remain unknown, but recent data indicate that VP24 possesses structural features consistent with viral matrix proteins and that it might have a role in viral assembly and budding.⁷⁸ VP24 and VP35 have been shown to play a role in interfering with type I IFN signaling (discussed below).

Flaviviridae

Flavivirus particles contain an approximately 11-kb, single, positive-stranded RNA genome. A single open reading frame is flanked by 5' and 3' noncoding regions and produces a large polyprotein that is cotranslationally and posttranslationally processed by cellular proteases into three structural proteins and seven non-structural proteins in the order C-prM/M-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5.^{79,80} The nucleocapsid is composed of a single capsid protein (C). In infected cells, the prM protein is cleaved by furin to form a small, nonglycosylated membrane protein (M) and an N-terminal "pr" segment that is secreted. E protein is a large glycosylated type I membrane protein. The remaining proteins are nonstructural proteins. The NS1 protein secreted from infected mammalian cells is thought to play a role in RNA replication.⁸¹ The function of NS2A is unknown, but some data suggest that it may function in the recruitment of RNA templates to the membrane-bound replicase, or it could be involved in the inhibition of IFN.^{82,83} NS2B is a small membrane-associated protein that forms a complex with NS3 and is a required cofactor of the serine protease function of NS3.⁸⁴ NS3, a large cytoplasmic protein that associates with membranes by interacting with NS2B, is thought to play a role in polyprotein processing and RNA replication.⁸⁴⁻⁸⁸ NS4A and NS4B are membrane-associated proteins; NS4A appears to be involved in RNA replication,^{82,88,89} and NS4B is also localized to sites of RNA replication and may be involved in inhibiting IFN signaling.⁹⁰ NS5 contains sequence homology similar to RNA-dependent RNA polymerases of other positive-stranded RNA viruses and also with methyltransferase enzymes involved in RNA cap formation.^{91,92}

CLINICAL MANIFESTATIONS

Patients infected with these viruses may experience a wide spectrum of clinical manifestations with varying degrees of severity, yet not all patients develop classic VHF syndrome. The exact nature of the disease depends on the viral virulence and strain characteristics, routes of exposure, dose, and host factors. For example,

DHF/DSS typically develops only in patients previously exposed to heterologous dengue serotypes.⁹³ As another example, for Ebola HF, the Zaire species is clearly more pathogenic in humans and nonhuman primates than the Sudan species, yet the incubation period reported for person-to-person transmission in

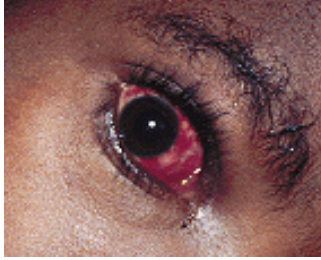


Fig 13-2. Ocular manifestations of viral hemorrhagic fever. Conjunctival injection and subconjunctival hemorrhage, as seen in this Lassa fever patient, are sometimes associated with viral hemorrhagic fever infection.

Photograph: Courtesy of Daniel G Bausch, MD, MPH&TM, Tulane School of Public Health and Tropical Medicine, New Orleans, Louisiana.

Ebola-Zaire infections greatly exceeds the incubation period for injections or needle stick accidents.⁹⁴

A main target organ in VHF syndrome is the vascular bed; correspondingly, the dominant clinical features are usually a consequence of microvascular damage and changes in vascular permeability.⁹⁵ Common presenting complaints are fever, myalgia, and prostration; clinical examination may reveal only conjunctival injection, mild hypotension, flushing, and petechial hemorrhages. Fulminant VHF typically evolves to shock and generalized bleeding from the

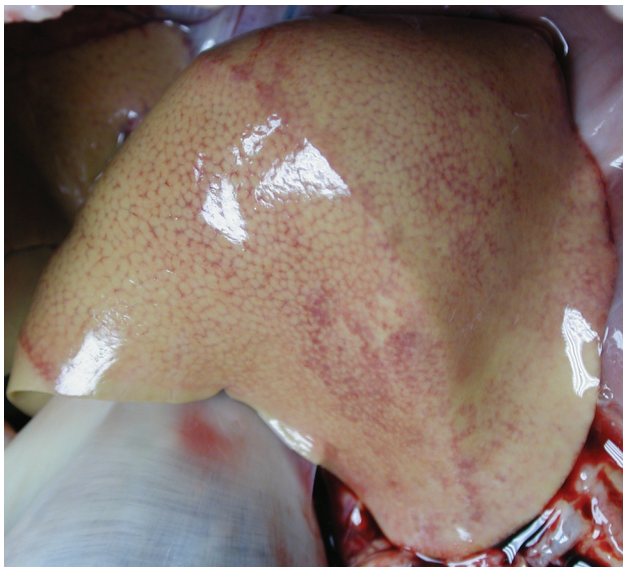


Fig 13-3. Liver pathology in viral hemorrhagic fever. Liver of a rhesus monkey experimentally infected with Marburg virus (Angola strain) showing diffuse reticulated pattern resulting from degeneration and necrosis.

mucous membranes (Figure 13-2), and often is accompanied by evidence of neurological, hematopoietic, or pulmonary involvement. Hepatic involvement is common (Figure 13-3), but only a small percentage of patients with RVF, CCHF, Marburg HF, and Ebola HF manifest a clinical picture dominated by jaundice and other evidence of hepatic failure. Renal failure is proportional to cardiovascular compromise, except for patients with HFRS caused by hantaviruses, in which renal failure is an integral part of the disease process and oliguria is a prominent feature of the acutely ill patient.⁹⁶ VHF mortality may be substantial, ranging from 5% to 20% or higher in recognized cases. Ebola and Marburg outbreaks in sub-Saharan Africa have had particularly high case-fatality rates, ranging from 50% to 90%.²³⁻²⁷

The overall incubation period for VHF varies from 2 to 35 days. There is a prodrome period that may include a high fever, headache, malaise, myalgias, arthralgia, abdominal pain, nausea, and diarrhea, which usually lasts less than a week. The clinical characteristics vary with the viral agent involved. Filoviruses, flaviviruses, and RVF tend to have an abrupt onset, whereas arenaviruses have a more insidious onset. For Lassa fever patients, hemorrhagic manifestations are not pronounced, and neurological complications are infrequent, develop late, and manifest only in the most severely ill group. Deafness is a frequent consequence of severe Lassa fever. For the South American arenaviruses (Argentine and Bolivian HFs), neurological and hemorrhagic manifestations are much more prominent. RVF virus is primarily hepatotropic and hemorrhagic disease is infrequent. In recent outbreaks in Egypt, retinitis was frequently associated with RVF virus infection.⁹⁷

Unlike RVF, in which hemorrhage is not prominent, infection with CCHF is usually associated with profound disseminated intravascular coagulation (DIC) (Figure 13-4). Patients with CCHF may bleed profusely, and because this occurs during the acute, viremic phase, contact with an infected patient's blood is a special concern. Several nosocomial outbreaks have been associated with CCHF virus.

The clinical picture for diseases caused by hantaviruses is evolving, especially now in the context of HPS. The pathogenesis of HFRS may be somewhat different; immunopathological events seem to be a major factor. When patients present with HFRS, they are typically oliguric. Surprisingly, the oliguria commences while the patient's viremia is resolving and patients are mounting a demonstrable antibody response. This occurrence has practical significance in that renal dialysis can be started with relative safety. Clinical data from human outbreaks caused by filoviruses are sparse. Although mortality is



Fig. 13-4. Massive cutaneous ecchymosis associated with late-stage Crimean-Congo hemorrhagic fever viral infection, 7 to 10 days after clinical onset. Ecchymosis indicates multiple abnormalities in the coagulation system, coupled with loss of vascular integrity. Photograph: Courtesy of Dr Sadegh Chinikar, Pasteur Institute of Iran, Tehran, Iran.

high, outbreaks are rare and sporadic. Marburg and Ebola viruses produce prominent maculopapular rashes in both human and nonhuman primates (Figure 13-5), and DIC appears to be a factor in their pathogenesis. Therefore, treating the DIC should be considered, if practicable, for these patients.

Among the flaviviruses, yellow fever virus is hepatotropic; black vomit caused by hematemesis has been associated with this disease. Patients with yellow fever develop clinical jaundice and die with something comparable to hepatorenal syndrome. Dengue HF and shock are uncommon, life-threatening complications of dengue, and are thought to result from an immunopathological mechanism triggered by sequential infections with different dengue viral serotypes (especially in children).⁹³ Although this is the general epidemiological pattern, dengue virus may also (rarely) cause HF during primary infections and in adults.⁹⁸ Laboratory findings for VHF may include



Fig. 13-5. Characteristic petechial rash of the abdomen and inguinal region of a cynomolgus monkey infected with Marburg virus. Note also the abnormalities in the coagulation system as evidenced by subcutaneous pooling of blood at a recent venipuncture site on animal's left inner thigh.

thrombocytopenia (or abnormal platelet function) or leukopenia (except for Lassa fever, which includes leukocytosis). Some patients have anemia, and others have hemoconcentration; most have elevated liver-associated enzymes. Bilirubin is elevated in RVF and yellow fever. Prothrombin time, activated partial thromboplastin time, and bleeding time are often prolonged. Patients in DIC have elevated fibrin degradation products and decreased fibrinogen. Urine tests may show proteinuria and hematuria; patients with renal failure may have oliguria or azotemia. Blood, occult or overt, may be present in stools.

PATHOGENESIS

Understanding the kinetics of host-pathogen relationships and identifying critical pathogenetic processes are important for the rational development of prophylactic and therapeutic countermeasures. For the most part, the specific mechanisms underlying the pathogenesis of HF viral infection have not been clearly explained, although recent progress has been made, particularly on Ebola virus. A paradigm showing the current views on the pathogenesis of the HF viruses is illustrated in Figure 13-6. A central theme common to

all VHF, with the possible exception of yellow fever, is that lesions are not severe enough to account for terminal shock and death of the host. Yet VHF infections are characterized by a fulminant shock-like syndrome in fatal cases, suggesting that inflammatory mediators may play a determining role in the disease pathogenesis. Fatal HF viral infections are generally characterized by high viremia and immunosuppression. HF viral infection in humans and nonhuman primates is characterized by deleterious changes in lymphoid

tissues and defects in the coagulation system. Another common feature among these viruses is that all of the HF viruses appear to target and impair the cells that play the most critical roles in initiating the antiviral

immune response, likely leading to unchecked and overwhelming viral burdens. To provide a better understanding of these pathogenic events, this section looks at the interactions between VHF and the cells

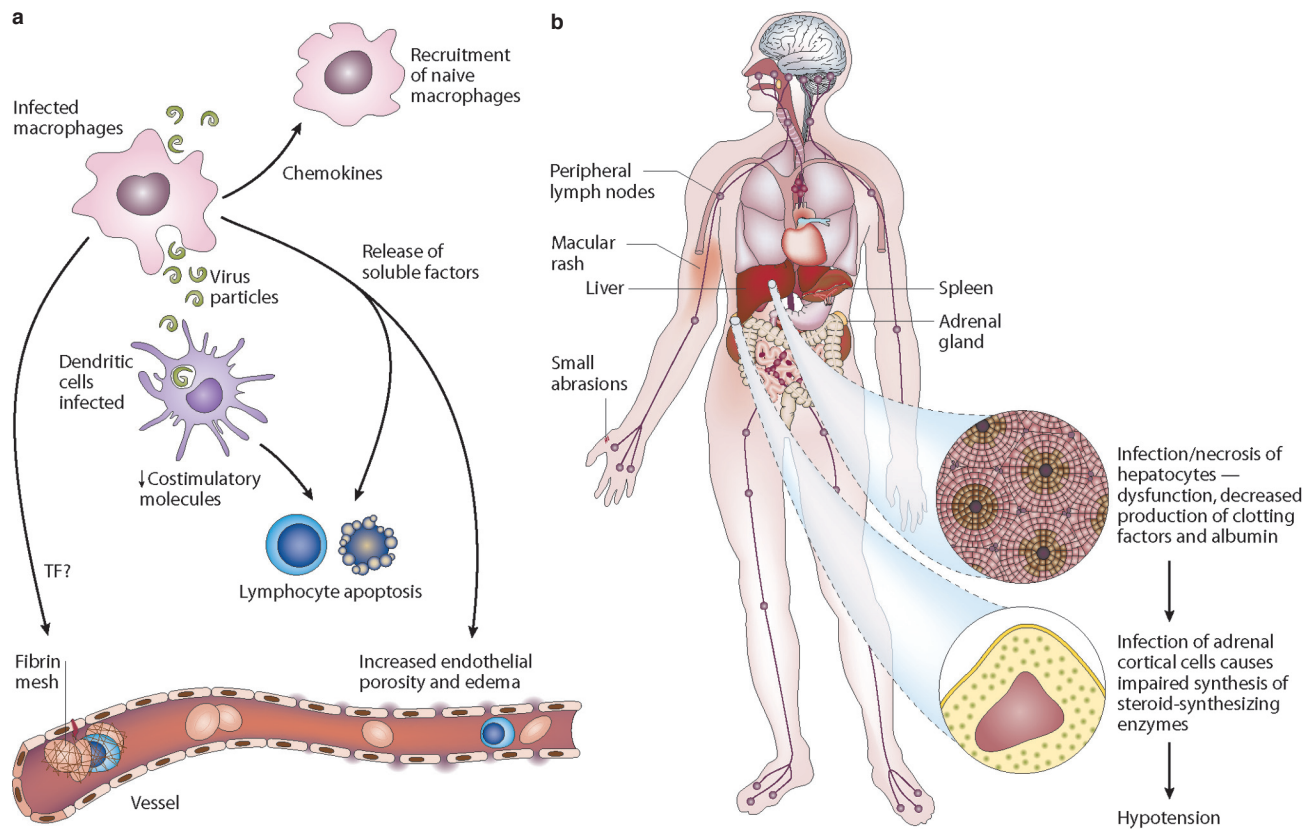


Fig. 13-6. Model of viral hemorrhagic fever (VHF) pathogenesis. **(a)** Virus spreads from the initial infection site to regional lymph nodes, liver, and spleen. At these sites, the virus infects tissue macrophages (including Kupffer cells) and dendritic cells. Soluble factors released from virus-infected monocytes and macrophages act locally and systemically. Release of chemokines from these virus-infected cells recruits additional macrophages to sites of infection, making more target cells available for viral exploitation and further amplifying the dysregulated host response. Although none of these viruses infects lymphocytes, the rapid loss of these cells by apoptosis is a prominent feature of disease. The direct interaction of lymphocytes with viral proteins cannot be discounted as having a role in their destruction, but the marked loss of lymphocytes is likely to result from a combination of factors, including viral infection of DC and release of soluble factors from virus-infected monocytes and macrophages. For example, viral infection of dendritic cells impairs their function by interfering with the upregulation of costimulatory molecules, which are important in providing rescue signals to T lymphocytes. Additionally, release of soluble factors from infected monocytes and macrophages results in deletion of lymphocytes, both directly by release of mediators such as nitric oxide, and indirectly by contributing to upregulation of proapoptotic proteins such as Fas and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). The coagulation abnormalities vary in nature and magnitude among the VHF. For example, Ebola virus induces the overexpression of tissue factor (TF) that results in activation of the clotting pathway and the formation of fibrin in the vasculature. For example, coagulation disorders are less marked in Lassa fever, and impairment of endothelial function contributes to edema, which seems to be a more prominent finding in Lassa fever than in other VHF. **(b)** The hemodynamic and coagulation disorders common among all of the VHF are exacerbated by infection of hepatocytes and adrenal cortical cells. Infection of hepatocytes impairs synthesis of important clotting factors. At the same time, reduced synthesis of albumin by hepatocytes results in a reduced plasma osmotic pressure and contributes to edema. Impaired secretion of steroid-synthesizing enzymes by VHF-infected adrenal cortical cells leads to hypotension and sodium loss with hypovolemia. Macular rashes are often seen in VHF. Reproduced with permission from Macmillan Publishers Ltd.: Geisbert TW, Jahrling PB. Exotic emerging viral diseases: progress and challenges. *Nat Med.* 2004;10(12 suppl):S110–121.

and tissues they affect as well as factors contributing to the immunological and hematological imbalances associated with HF viral infection. Dengue HF is not addressed in this section because of its complex nature (partly attributable to antibody-dependent enhancement), which does not appear to play a prominent role in other HF viral infections. Several researchers have reviewed the pathogenesis of dengue HF.⁹⁹⁻¹⁰²

Target Cells and Tissues

In general, the HF viruses all have a broad cell tropism, infecting a wide range of cell types. Immunohistochemistry and in situ hybridization analyses of tissues from fatal human cases or experimentally infected nonhuman primates show that monocytes, macrophages, dendritic cells (DCs), endothelial cells, hepatocytes, and adrenal cortical cells all generally support replication of these viruses.¹⁰³⁻¹¹⁶ The sequence of infection, however, is largely unknown. Systematic temporal studies in nonhuman primates experimentally infected with Ebola-Zaire virus suggest that monocytes, macrophages, and DCs are early and preferred targets of these viruses, whereas endothelial cells are infected much later during the course of disease, proximal to death.^{114,115} Infection of endothelial cells appears to play a larger role in the pathogenesis of the hantaviruses than of the other HF viruses; although endothelial damage probably does not occur by direct effects of hantaviral replication.^{117,118}

The mechanism (or mechanisms) of entry of the HF viruses into host cells has not been well characterized, but it is not believed to occur by direct fusion with the plasma membrane. Instead, researchers think that these viruses exploit the host cell's endocytic machinery to access the cytoplasm. Many different types of cell-surface binding proteins have been proposed to play a role in the entry of the viruses that cause VHF. For example, the asialoglycoprotein receptor of hepatocytes is postulated to serve as a binding protein for the Marburg virus,¹¹⁹ whereas the folate receptor α and the DC-specific intercellular adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN) and DC-SIGN-related factors have also been associated with the entry of the Marburg virus and the Ebola virus.¹²⁰⁻¹²⁵ Moreover, the $\beta 1$ integrin receptors and a human macrophage galactose- and *N*-acetylgalactosamine-specific C-type lectin are associated with the entry of Ebola virus.¹²⁴⁻¹²⁵ $\beta 3$ integrins might mediate entry of the several hantaviruses.¹²⁶ Alpha-dystroglycan was identified as an important receptor for Lassa virus,¹²⁷ but does not appear to be a receptor for the South American arenaviruses that cause HF.^{128,129} Again, because these viruses have such a broad cell tropism,

infecting a wide range of cell types, it is highly likely that they exploit many molecules for entry. Consistent with this notion, it has been proposed that Ebola virus uses a variety of different C-type lectins for efficient entry into host cells.¹²⁵

The similar cell and tissue tropism among the VHFs suggests commonalities in the entry mechanisms. Findings in many laboratories have shown that the transmembrane proteins of many RNA viruses including Ebola and Lassa have common structural and functional elements essential for viral entry.¹³⁰ For example, these viruses share a coiled-coil type of entry protein. Researchers anticipate that these general principles may also apply to other VHFs.

The role of the endothelium in the pathogenesis of the VHFs has been a particularly controversial topic. Vascular damage can be induced by immunological mechanisms and/or by direct infection of the vascular tissue. Impairment of endothelial cell functions can cause a wide range of vascular effects that lead to changes in vascular permeability or hemorrhage. Several in-vitro and ex-vivo studies have suggested that the Ebola virus GP is cytotoxic and is a main determinant of vascular cell injury, thus implying that direct Ebola virus-replication-induced structural damage of endothelial cells triggers the hemorrhagic diathesis.^{131,132} However, more recent in-vitro studies suggest that cell rounding and downregulation of surface markers are late events in Ebola infection, whereas synthesis and massive release of virions occur at early steps and do not cause significant cytotoxic effects.¹³³ These in-vitro findings are supported by in-vivo studies showing that Ebola infection of endothelial cells does not trigger cell death and that endothelial cell infection occurs only late in the disease course.¹¹⁵ Likewise, in-vitro studies have shown that Lassa virus can replicate in human endothelial cells without damaging them.¹³⁴

Scientists searched for the etiology of the hemorrhagic diatheses in fatal cases caused by Ebola virus and Marburg virus in tissues from the initial outbreaks in 1967 and 1976, respectively, but no vascular lesions were identified.^{135,136} In a recent study, consistent with the original histology observations in fatal human cases, Geisbert and colleagues demonstrated that Ebola virus infection of endothelial cells does not extensively disrupt the architecture of the vascular endothelium in Ebola-infected cynomolgus monkeys.¹¹⁵ As noted previously, although Ebola virus replicated in endothelial cells of these animals, endothelial cell infection was only seen focally at late stages of disease, after the onset of the hemorrhagic abnormalities that characterize Ebola HF. Although ultrastructural evidence of endothelial cell activation and disruption was observed at midpoint to end stages of disease, it was postulated that the vasoac-

tive effects on endothelial cells were mediated indirectly because these changes were not associated with the presence of intracytoplasmic Ebola viral antigens.¹¹⁵ Feldmann and colleagues support the view that mediator release from filovirus-infected target cells can have deleterious effects on the endothelium.¹³⁷

For other VHF, endothelium may be affected in a manner similar to the paradigm presented for the filoviruses. No specific vascular lesions were found in 12 fatal cases of AHF,¹³⁸ nor were specific vascular lesions observed in rhesus monkeys experimentally infected with Machupo virus.¹³⁹ Endothelium was only minimally infected in rhesus monkeys experimentally infected with Lassa virus, and overt endothelial necrosis was not observed histologically.¹⁴⁰ As noted previously, the endothelium appears to play a more important role in hantavirus infections than in other VHF; however, capillary leakage caused by hantavirus infection is thought to occur as a consequence of immune-mediated endothelial injury and not by direct effects of viral replication.^{117,118}

In addition to the macrophage-rich lymphoid tissues, the liver and the adrenal gland appear to be important target organs for all HF viruses, and this tropism likely plays an equally important role in the disease pathogenesis. Various degrees of hepatocellular necrosis were reported in HF viral infections of humans and nonhuman primates^{109,114,136,139,141-145}; however, as noted before, the hepatocellular lesions are generally not significant enough to explain the cause of death. The exception is yellow fever, in which the extent of direct liver injury in some cases is severe enough to account for the disease. Markers of hepatocellular injury and fulminant hepatic dysfunction such as circulating liver-associated enzymes (eg, aspartate aminotransferase, alanine aminotransaminase) directly correlate with severity of yellow fever infection and prognosis.¹⁴⁶ Elevations in liver-associated enzymes are prominent findings in most severe VHF infections.^{6,22,70,105,114,116,147-155} Hemorrhagic tendencies could be related to decreased synthesis of coagulation and other plasma proteins resulting from severe hepatocellular necrosis. In addition, reduced synthesis of albumin may cause a reduction in plasma osmotic pressure and contribute to edema, which again appears to be a recurrent feature of severe cases of Lassa fever.^{156,157}

Various degrees of adrenocortical infection and necrosis were reported in HF viral infections of humans and nonhuman primates.^{105,111,114,144,158} The adrenal cortex plays an important role in controlling blood pressure homeostasis. Impaired secretion of steroid-synthesizing enzymes leads to hypotension and sodium loss with hypovolemia, which are important elements that have been reported in nearly all cases of

VHF.^{70,116,141,149,159-161} This finding suggests that impairment of adrenocortical function by viral infection may play a particularly important role in the evolution of shock that typifies late stages of VHF.

Immunosuppression

For nearly all VHF, various degrees of lymphoid depletion and necrosis are seen in spleen and lymph nodes of fatal cases and in experimentally infected nonhuman primates (Figure 13-7).^{103,104,112,114,116,135,139,142,143,149,150,162-169} Although lymphoid tissues are principal targets for HF viral infection, there is usually little inflammatory cellular response in these tissues or other infected tissues. With the exception of the hantaviruses, lymphopenia appears to be the most consistent pathological finding among HF viral infections of humans and nonhuman primates.^{109,114,152,162,167,169-179} Despite the significant loss of lymphocytes during HF viral infection, none of the HF viruses replicates in lymphocytes. For Ebola and Marburg viruses, large numbers of lymphocytes undergo apoptosis in humans and experimentally infected nonhuman primates,^{114,180-182} partly explaining the progressive lymphopenia and lymphoid depletion at death. The prominence of tingible body macrophages in lymphoid tissues of rhesus monkeys experimentally infected with Junin virus suggests that apoptosis is also a primary factor in the loss of lymphocytes noted for other VHF.¹⁴⁵

The mechanism (or mechanisms) for the underlying apoptosis and loss of bystander lymphocytes during

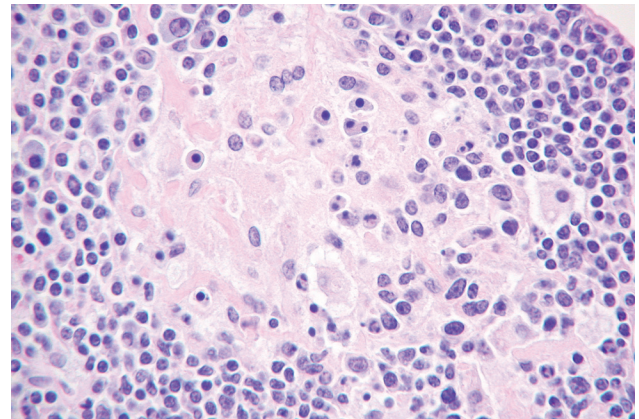


Fig. 13-7. Lymphoid depletion in viral hemorrhagic fever. Tonsil from a rhesus monkey experimentally infected with Ebola-Zaire virus, showing hyalinized follicle with typical depletion and apparent apoptosis of lymphocytes (hematoxylin-eosin stain, original magnification $\times 40$). Photograph: Courtesy of Dr Kelly Davis, Charles River Laboratories, Redfield, Arkansas.

the course of VHF illness has been unclear, but it is likely induced through multiple pathways. These pathways or processes may include the tumor necrosis factor-related, apoptosis-inducing ligand (TRAIL) and Fas death receptor pathways,^{114,183} dysfunction of DCs induced by HF viral infection,^{114,183-186} and abnormal production of proapoptotic soluble mediators such as nitric oxide (NO).^{114,179,183,187}

Inflammatory Response

Cytokines, chemokines, and other inflammatory mediators function in a pleiotropic manner, acting on many different types of cells to modulate the host's immune response. Although cytokines and chemokines typically apply their antimicrobial actions locally, for example in areas of infection, cytokines and chemokines might also act systemically, and they commonly induce many of the symptoms of infection (eg, fever, myalgia). When present in high concentrations, cytokines and chemokines can have toxic or even lethal effects; studies of septic shock have associated abnormal production of pro-inflammatory cytokines and chemokines with disease severity and fatal outcome.¹⁸⁸⁻¹⁹¹

HF viral infection of humans and nonhuman primates triggers the expression of many inflammatory mediators, including the IFNs; interleukin (IL)-6; IL-8; IL-10; IL-12; IFN-inducible protein-10; monocyte chemoattractant protein-1; regulated upon activation, normal T-cell expressed and secreted (RANTES); tumor necrosis factor- α , and reactive oxygen and nitrogen species.^{6,114,179,180,183,187,192-205} Infection of many different primary human cells in vitro also shows that HF viral infection can trigger the production of many of these same inflammatory mediators.^{115,137,183,206-210} Overall, it appears that virus-induced expression of these mediators results in an immunological imbalance that in part contributes to the disease progression. However, information regarding the inflammatory response after infection with the HF viruses is incomplete, and the existing data are often inconsistent. For example, high levels of IFN- α were reported in acute phase sera of patients infected with Ebola virus in one study¹⁹⁹ but not detected in a subsequent yet similar study.¹⁸⁷ Such contradictions also confound interpretation of some in-vitro data. The differences in profiles of circulating cytokines and chemokines among the VHFs may be attributable to factors other than the differences among the viruses, such as genetic differences among patients and, in particular, differences related to the disease phase when the samples were obtained.

Researchers have postulated that for patients with asymptomatic, nonfatal Ebola virus infection, the infection is controlled by an initial increase in cytokines,

including IL-1 β , IL-6, and tumor necrosis factor- α , which is followed by a return to preexposure levels.²¹¹ Protection against fatal outcome for other VHFs may likewise depend on an early and robust cytokine response, but this remains to be established. Conversely, disease severity may also be increased by an inappropriate proinflammatory response early in the disease course. Thus, the delicate balance between protective and deleterious cytokine and chemokine responses remains to be defined for all the VHF agents.

In general, the type I IFN response appears to play an important role in the pathogenesis of the HF viruses, especially for RVF. A delayed IFN response was correlated with increased mortality in a rhesus monkey model of RVF.¹⁵² In AHF patients, circulating levels of IFN- α are unusually high and during the acute phase are significantly higher in fatal cases than in survivors.¹⁹³ As noted previously, inconsistencies were reported in circulating levels of IFN- α in Ebola-infected patients; however, very high plasma levels of IFN- α were observed in experimentally infected monkeys.^{114,183} The role of IFNs in Lassa fever is unclear. Several studies have evaluated resistance of Lassa virus to IFNs in vitro. In one study, Lassa was shown to be resistant to IFN- α ,²¹² and in a more recent study, IFN- α and IFN- γ were shown to inhibit the replication of Lassa virus.²¹³ Less is known about the role of IFNs in other VHFs, although a recent study suggested that IFN- α inhibits CCHF in vitro.²¹⁴ A significant concern when interpreting and comparing results from any of these VHF studies is the observation that there are 12 different subtypes of IFN- α , and previous studies have shown that the antiviral activities of the subtypes vary greatly.²¹⁵ The research on HF viruses has evaluated only total levels of IFN- α , and no researchers have dissected out which subtypes are represented in the plasma of infected patients and which subtypes may or may not have antiviral properties.

The ability of these viruses to directly modulate the host inflammatory response has yet to be fully delineated. Again, more research has been done on Ebola virus than any other VHF. The Ebola viral protein VP35 reportedly functions as a type I IFN antagonist.²¹⁶⁻²¹⁸ Recent studies show that VP35 prevents IFN regulatory factor activation by inhibiting phosphorylation, and it is likely that VP35 prevents transcription of IFN- β .²¹⁷ Other studies suggest that Ebola viral protein VP24 expression might also interfere with type I IFN signaling.^{218,219} RVF virus also has a viral protein, NSs, that functions as a type I IFN antagonist.²²⁰ Based on studies with dengue virus, researchers think that flaviviruses including yellow fever virus inhibit type I IFN signaling primarily by the NS4B protein.^{90,221} Little is known about whether other VHFs possess analogous

proteins that interfere with type I IFNs, but this possibility merits further study.

Although not extensively reported, one consistency in the proinflammatory response among the VHFs is the potential importance of reactive oxygen and nitrogen species in the disease pathogenesis. Increased blood levels of NO were reported in nonhuman primates experimentally infected with Ebola virus^{114,183} and recently confirmed in Ebola-infected patients.^{179,187} Sanchez and colleagues associated increased blood levels of NO with mortality.¹⁷⁹ Significant blood levels of NO have also been demonstrated in AHF patient sera²⁰⁹ and in patients infected with hantaviruses.^{195,202} Abnormal NO production has been associated with many pathological conditions, including apoptosis of bystander lymphocytes (as noted previously), tissue damage, and loss of vascular integrity, which may contribute to virus-induced shock. NO is known to have both protective and caustic effects, and this autotoxic overproduction may represent the host's endogenous counter-regulatory mechanism of protection against noxious agents, in this case the VHF viruses. In general, microbes induce monocytes and macrophages to produce NO in an attempt to control infection. However, in the case of the HF viruses, monocytes and macrophages are preferred target cells for viral replication. Enhanced replication in these cells may in turn exacerbate disease by producing large amounts of NO, resulting in deleterious effects, such as suppressive effects on lymphocyte proliferation and damage to other cells. NO is an important mediator of hypotension,^{222,223} and, as noted previously, hypotension is a prominent finding among most of the VHFs.^{70,116,141,149,159-161} Together, the information collected suggests that an impaired and ineffective immune response leads to high levels of virus and proinflammatory mediators in the late stages of disease, which is important in the pathogenesis of hemorrhage and shock in VHFs.

Coagulation Abnormalities

Abnormalities in blood coagulation and fibrinolysis during VHF infection are manifested as petechiae, ecchymoses, mucosal hemorrhages, and uncontrolled bleeding at venipuncture sites. However, massive loss of blood is atypical and, when present, is largely restricted to the gastrointestinal tract. Even in these cases, blood volume loss is insufficient to account for death. DIC is a syndrome characterized by systemic intravascular activation of coagulation leading to widespread deposition of fibrin in the vasculature, which contributes to the development of multiple organ failure.²²⁴⁻²²⁶ DIC is associated with both bleeding and thrombotic abnormalities, and widespread thrombosis

and bleeding commonly occur simultaneously. The occurrence of DIC in HF viral infection is the subject of much debate, and information that supports or refutes the presence of DIC is inconclusive. In general, DIC appears to be more prominent in Ebola HF and CCHF than among the other VHFs. The presence of DIC in any of the VHFs appears to strongly correlate with a poor outcome. For the purposes of this review, the authors can clearly state that regardless of whether DIC is an important and consistent feature among all VHFs, impairment of the coagulation system ostensibly contributes to the disease pathogenesis of all of these VHFs. Both coagulation and fibrinolysis appear to be activated by HF viral infection, and the degree of impairment of the coagulation system seems to be associated with the balance between these counteracting processes by the host.

Most VHF infections in humans and in nonhuman primate models are characterized by cutaneous flushing or macular rashes; however, the characteristics of these rashes vary among the VHFs. For example, nonpruritic petechial skin rashes on the axillae and groins, forehead, and chest appear in up to 50% of patients infected with Ebola or Marburg viruses and are more evident in patients with light-colored skin.^{22,25-27,227,228} This same type of rash evolves in more than 50% of nonhuman primates (of the genus *Macaca*) experimentally infected with Ebola or Marburg viruses.^{109,114,153,171,173,229,230} Petechial skin rashes are also associated with yellow fever.¹¹⁶ In general, arenavirus infections in humans and in nonhuman primate models are typically characterized by flushed, erythematous rashes on the face and thorax^{139,145,231,232}; although oral and axillary petechia are frequently observed in human cases of AHF.²³³ For many VHFs, petechiae are sometimes observed on visceral organs.^{114,116,140} In addition, congestion of various organs is a frequent finding at autopsy or necropsy.^{109,114,141,143,156,165}

Thrombocytopenia appears to be a consistent finding among VHF infections of humans and nonhuman primates,^{10,22,108,116,135,138,141,151,152,155,160,168,170,172,173,179,230,234-239} with the notable exception of Lassa fever.^{105,140,162,240,241} Moderate thrombocytopenia was reported in patients with severe Lassa fever, but the most significant changes were noted in platelet function, which was markedly depressed in these patients.¹⁴⁹ Marked changes in platelet function have also been observed in Lassa-infected rhesus macaques¹⁶² and in rhesus monkeys experimentally infected with yellow fever.¹¹⁶ Researchers have postulated that the thrombocytopenia seen in the South American VHFs results in part from maturation arrest of megakaryocytes attributable to the high levels of IFN in these patients.¹⁹³ Similar inferences have been made for yellow fever.¹¹⁶

Histological and biochemical evidence of impairment of the coagulation system has been shown for many VHFs, but the data are largely incomplete and paradoxical. More is known about Ebola and Marburg viruses than the other VHFs. Fibrin deposition has been documented at autopsy for Marburg HF,^{136,242} and clinical laboratory data suggest that DIC is an important feature of human Ebola and Marburg HF.^{25,243} Numerous studies have shown histological and biochemical evidence of DIC during Ebola infection in a variety of nonhuman primate species, including significant changes in markers of blood coagulation and fibrinolysis, such as various clotting factors, fibrin degradation products (FDPs), D-dimers, protein C, tissue plasminogen activator, and urokinase plasminogen activator.^{109,114,153,171,230,239} Fibrin and fibrinocellular thrombi in vessels in numerous tissues and fibrin deposits in the red pulp and marginal zone of the spleen are frequent findings in Ebola virus-infected cynomolgus and rhesus macaques.^{107,109,230,239,244}

AHF infections are characterized by significant changes in markers of blood coagulation and fibrinolysis, including thrombin-antithrombin complexes, prothrombin fragments, protein C, D-dimers, tissue plasminogen activator, and plasminogen activator inhibitor-1.²³⁷ Increased fibrinogen levels were detected in a study of 32 AHF patients, but FDPs were not detected, and DIC did not appear to be a relevant factor in these cases.²³³ Three of 12 AHF cases showed intravascular fibrin thrombi and clinical features consistent with DIC.¹³⁸ Neotropical primates experimentally infected with Junin virus showed an increase in the prothrombin time and increases in circulating levels of fibrinogen and FDPs.²³⁶ Prolongation of the activated partial thromboplastin time was noted in rhesus monkeys experimentally infected with Machupo virus, but evidence for DIC was inconclusive because of equivocal changes in fibrinogen levels and levels of FDPs.²³⁵ Microscopic evidence of DIC was noted in only 1 of 10 rhesus monkeys experimentally infected with Machupo virus.¹³⁹

Evidence of DIC has been reported in fatal cases of CCHF. Values for prothrombin ratio, prothrombin time, activated partial thromboplastin time, and FDPs were grossly elevated in 15 fatal cases, but fibrinogen and hemoglobin levels were depressed.¹⁵¹ Many of these clinical pathologic changes were evident at an early stage of disease and had a highly predictive value for fatal outcome in the 35 monitored cases. Experimental infection of rhesus monkeys with RVF virus does not produce a uniformly lethal disease. However, as in human cases, the degree of hemorrhagic manifestations is associated with fatal outcome. Not surprisingly,

changes in circulating levels of clotting factors, activated partial thromboplastin time, prothrombin time, FDPs, and evidence of DIC were more prominent in 3 of 17 RVF virus-infected monkeys that succumbed to challenge than animals that survived.^{150,152} Multiple fibrin thrombi were present within the glomeruli and small intertubular vessels of these experimentally infected rhesus monkeys. In addition, fibrillar material that stained positive for fibrinogen was abundant in the spleen. Biochemical evidence of DIC has been noted in about half of the cases of Korean HF,²⁴⁵ and microscopic evidence of alveolar fibrin was reported in cases of HPS.²⁴⁶ DIC also appears to play a role in yellow fever; changes in clotting and prothrombin times, clotting factors, fibrinogen levels, and FDPs have been reported.^{116,247}

DIC does not appear to be involved in Lassa fever. Microscopic hemorrhagic diathesis is rare, and the absence of fibrin thrombi correlates with generally normal measurements of coagulation mechanisms.^{140,240} In one report, however, splenic necrosis that centered in the marginal zone was accompanied by the deposition of fibrin.¹⁴⁰ In rhesus monkeys experimentally infected with Lassa virus, several groups reported no changes in circulating levels of clotting factors and no evidence of DIC.^{105,162,241}

The mechanism (or mechanisms) for triggering the coagulation abnormalities seen in VHF has not been fully delineated. Some of the latest studies on Ebola virus have begun to shed light on the pathogenesis of coagulation system dysregulation and suggest that development of coagulation abnormalities might occur much earlier than previously thought. Although it is likely that the coagulopathy seen in Ebola HF is caused by multiple factors, particularly during the later stages of disease, recent data strongly implicate tissue factor expression/release from Ebola-infected monocytes and macrophages as a key factor that induces the development of coagulation irregularities.²³⁹ Levels of expression of tissue factor may also be affected by the production of proinflammatory cytokines, which (as noted previously) are induced in most HF viral infections. For example, IL-6 has been shown to effectively upregulate expression of tissue factor on monocyte^{248,249} and endothelial cells.²⁵⁰ Ruf recently reviewed the role of tissue factor in VHFs.²⁵¹ Other factors speculated to contribute to the coagulopathy seen in Ebola HF include impairment of the fibrinolytic system as evidenced by rapid declines in plasma levels of protein C during the course of infection in cynomolgus monkeys.²³⁹ Reduced plasma levels of protein C were also seen in AHF patients.²³⁷ Future studies are needed to further define and clarify the role of tissue factor and the protein C system in VHF.

Future Directions in Pathogenesis

Several recent developments in biomolecular technology will play major roles in future studies designed to elucidate the molecular mechanisms of these devastating diseases. One key breakthrough has been the successful development of reverse genetics systems for the generation of many HF viruses including infectious Ebola virus,^{252,253} Marburg virus,²⁵⁴ CCHF virus,²⁵⁵ RVF virus,²⁵⁶ and yellow fever virus.²⁵⁷ These infectious clone systems will have a tremendous impact on the ability to identify key regulatory elements and structure–function relationships in the HF viral life cycles. Another technology that will facilitate the ability to dissect the pathogenesis of HF viral infection is cDNA microarrays. A genomic view of systemic interactions that occur during HF viral

infection will provide clues to important host–virus interactions. A recent application of cDNA microarrays to experimental Ebola virus infections in non-human primates revealed prominent induction of NFκ-β and tumor necrosis factor-α-regulated genes for Ebola, in contrast to negligible expression during variola virus infection.²⁵⁸ Similarly, another recent microarray analysis demonstrated that Ebola and Marburg virus infection of human liver cells *in vitro* resulted in changes in expression of many genes associated with the immune system, coagulation, and acute-phase proteins.²⁵⁹ Comparative analysis among the VHFs, which may reveal differentially expressed genes unique to each agent, could have diagnostic utility by identifying markers of disease progression and predictors of outcome, as well as improving the understanding of disease pathogenesis.

DIAGNOSIS

Differential Diagnosis

In the event of a covert bioterrorist attack, a high degree of suspicion would be required for any realistic chance of rapid VHF diagnosis. Whether clinicians would initially recognize VHF is unclear, but a cluster of such cases would likely alert clinicians to this possibility. Under natural conditions, these viruses have a geographically restricted distribution linked to the ecology of the reservoir species and vectors; thus, a detailed travel history is critical in making the VHF diagnosis. Patients with arenaviral or hantaviral infections often recall seeing rodents during the presumed incubation period; however, as the viruses spread to humans by aerosolized excreta or environmental contamination, actual contact with the reservoir is not necessary. Large mosquito populations are common during the seasons when RVF virus and the flaviviruses are transmitted, but a history of mosquito bite is sufficiently common to be of little assistance in making a diagnosis, whereas tick bites or nosocomial exposures are of some significance when CCHF is suspected. History of exposure to animals in slaughterhouses should raise suspicions of RVF and CCHF in a patient with VHF.

The variable clinical presentation of these diseases presents a major diagnostic challenge. VHF should be suspected in any patient presenting with a severe febrile illness and evidence of vascular involvement (subnormal blood pressure, postural hypotension, petechiae, hemorrhagic diathesis, flushing of the face and chest, nondependent edema), who has traveled to an endemic area or to someplace where intelligence suggests a biological warfare or terror threat. Signs

and symptoms suggesting additional organ system involvement are common (headache, photophobia, pharyngitis, cough, nausea or vomiting, diarrhea, constipation, abdominal pain, hyperesthesia, dizziness, confusion, and tremor), but they rarely dominate the picture. The macular eruption characteristic of Marburg and Ebola HFs has considerable clinical importance.

As previously noted, laboratory findings can be helpful, although they vary from disease to disease, and summarization is difficult. Leukopenia may be suggestive, but in some patients, white blood cell counts may be normal or even elevated. Thrombocytopenia is a component of most VHF diseases, but to a varying extent. In some patients, platelet counts may be near normal, and platelet function tests are required to explain the bleeding diathesis. A positive tourniquet test has been particularly useful in diagnosing dengue HF, but this sign may be associated with other VHFs as well. Proteinuria or hematuria or both are common in VHF, and their absence virtually rules out AHF, Bolivian HF, and HFRS. Hematocrits are usually normal, and if there is sufficient loss of vascular integrity, perhaps mixed with dehydration, hematocrits may be increased. Soluble cytosolic liver-associated enzymes such as aspartate aminotransferase are frequently elevated. HF viruses are not primarily hepatotropic, but the liver is involved, and an elevated aspartate aminotransferase may help to distinguish VHF from a simple febrile disease.

For much of the world, the major differential diagnosis is malaria. Parasitemia in patients partially immune to malaria does not prove that malaria is responsible for symptoms. Typhoid fever, rickettsial,

and leptospiral diseases are major confounding infections; nontyphoidal salmonellosis, shigellosis, relapsing fever, fulminant hepatitis, and meningococemia are some of the other important diagnoses to exclude. Establishing the cause of DIC is difficult and often confusing. Many conditions that cause DIC, such as acute leukemia, lupus erythematosus, idiopathic or thrombotic thrombocytopenic purpura, and hemolytic uremic syndrome, could be mistaken for VHF.

Specific Diagnosis

Definitive diagnosis in an individual case rests on specific virological diagnosis. Most patients have readily detectable viremia at presentation (the exception is those with hantaviral infections). Infectious virus and viral antigens can be detected and identified by many assays of fresh or frozen serum or plasma samples or whole blood. Likewise, early immunoglobulin M antibody responses to the VHF-causing agents can be detected by enzyme-linked immunosorbent assays (ELISAs), often during the acute illness. Diagnosis by viral cultivation and identification requires 3 to 10 days for most VHFs (longer for the hantaviruses); with the exception of dengue, specialized microbiologic containment is required for safely handling these viruses.²⁶⁰ Appropriate precautions should be observed in collecting, handling, shipping, and processing diagnostic samples (see "Packaging Protocols for Biological Agents/Diseases" at <http://www.bt.cdc.gov/agent/vhf/index.asp>). Both

the Centers for Disease Control and Prevention and the US Army Medical Research Institute of Infectious Diseases (USAMRIID, Fort Detrick, Maryland) have diagnostic laboratories operating at the maximum biosafety level 4. Virus isolation should not be attempted without biosafety level 4 containment.

In contrast, most antigen-capture and antibody-detection ELISAs for these agents can be performed with samples that have been inactivated by treatment with beta-propiolactone²⁶¹ or gamma rays. Diagnostic tests based on reverse transcriptase polymerase chain reaction (RT-PCR) technology are safely performed on samples after RNA extraction using guanidium-based solutions. RT-PCR has been successfully applied to the real-time diagnosis of most of the VHF agents and is now the most widely used assay for identifying suspected VHF.²⁶²⁻²⁶⁶ Recently, a multiplex PCR assay in which microbial gene products are coded by a library of 64 distinct mass tags was developed and shown to be capable of differentiating 10 different agents of VHF.²⁶⁷ RT-PCR is particularly useful in cases where isolation of the infectious virus is difficult or impractical. RT-PCR has proven to be extremely valuable, for example, with HPS, in which Sin Nombre virus was recognized by PCR months before it was finally isolated in culture.²⁰ In cases where the identity of an agent causing suspected VHF is completely unknown, isolation in cell culture and direct visualization by electron microscopy, followed by identification by immunohistochemical procedures is frequently successful.^{1,268} Filoviruses and arenavi-

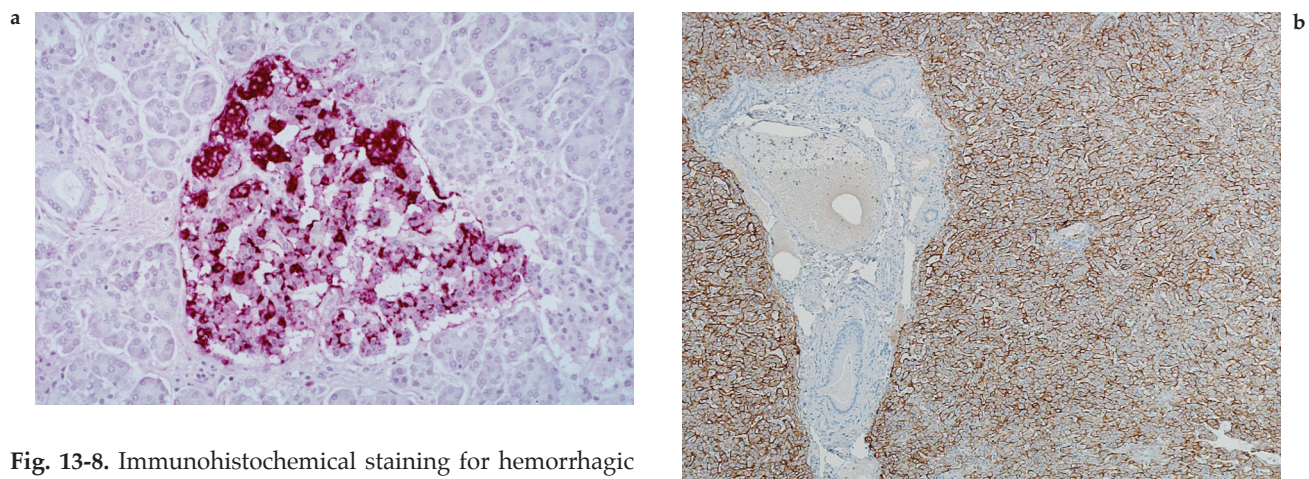


Fig. 13-8. Immunohistochemical staining for hemorrhagic fever viral antigens. (a) Pancreas from a fatal human case of Marburg hemorrhagic fever (Ravn strain). Note that Marburg virus–positive staining (red) is limited to the pancreatic islet, with multifocal distribution within the islet. (Streptavidin-alkaline phosphatase method, section counterstained with hematoxylin; original magnification $\times 20$.) (b) Liver of a rhesus monkey experimentally infected with Marburg virus (Angola strain). Note intense and widespread Marburg virus–positive staining (brown) of hepatocytes with little immunostaining in portal area. (Immunoperoxidase method, original magnification $\times 10$.)
Photograph b: Courtesy of LTC Tom Larsen, Pathology Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

ruses induce intracytoplasmic viral inclusions that are morphologically unique to each viral family. Moreover, Ebola and Marburg viruses can be distinguished from each other by their distinctive viral inclusion material²⁶⁹; trained microscopists can distinguish these filoviral genera by the size and shape of the viral particles.²⁶⁹ Immunohistochemical stains can be used to detect HF

viruses in tissue sections. The application of immunohistochemical stains to skin specimens can provide a comparatively rapid diagnosis.²⁷⁰ Immunohistochemical techniques are also useful for retrospective diagnosis of formalin-fixed tissues, where viral antigens can be detected and identified using batteries of specific immune sera and monoclonal antibodies (Figure 13-8).

MEDICAL MANAGEMENT

Patients with VHF syndrome require close supervision, and some require intensive care. Because the pathogenesis of VHF is not entirely understood and availability of antiviral drugs is limited, treatment is largely supportive. This care is essentially the same as the conventional care given to patients with other causes of multisystem failure. The challenge with VHF patients is to provide this support while minimizing the risk of infection to other patients and medical personnel.

Supportive Care

Patients with VHF syndrome generally benefit from rapid, atraumatic hospitalization to prevent unnecessary damage to the fragile capillary bed. Transporting these patients, especially by air, is usually contraindicated because of the effects of drastic changes in ambient pressure on lung water balance. Frequently patients manifest restlessness, confusion, myalgia, and hyperesthesia; these conditions should be managed by reassurance and other supportive measures, including the judicious use of sedatives, pain relief, and amnestic medications.

Secondary infections are common and should be sought and aggressively treated. Concomitant malaria should be treated aggressively with a treatment regimen known to be effective against the geographical strain of the parasite; however, the presence of malaria, particularly in immune individuals, should not preclude management of the patient for VHF syndrome if such treatment is clinically indicated.

Intravenous lines, catheters, and other invasive techniques should be avoided unless they are clearly indicated for appropriate management of the patient. Attention should be given to pulmonary toilet, the usual measures to prevent superinfection, and the provision of supplemental oxygen. Treatment with steroids or other agents that cause generalized immunosuppression has no empirical basis and is contraindicated, except possibly in treatment of HFRS.

The diffuse nature of the vascular pathological process may lead to a requirement for support of several organ systems. Myocardial lesions detected

at autopsy reflect cardiac insufficiency antemortem. Pulmonary insufficiency may develop, and, particularly with yellow fever, hepatorenal syndrome is prominent.³⁵

Treatment of Bleeding

The management of bleeding in VHF cases is controversial. Uncontrolled clinical observations support vigorous administration of fresh frozen plasma, clotting factor concentrates, and platelets, as well as early use of heparin for prophylaxis of DIC. In the absence of definitive evidence of VHF disease or DIC, mild bleeding manifestations should not be treated. More severe hemorrhage requires appropriate replacement therapy. When there is definitive laboratory confirmation of DIC, heparin therapy may be considered if appropriate laboratory support is available. Supportive strategies directed toward inhibiting coagulation activation may be warranted and have been shown to be beneficial in experimental and initial clinical studies.²⁷¹ Many new modalities to manage the pronounced coagulopathy that typifies many VHFs are being evaluated, most notably in nonhuman primate models of Ebola HF (discussed below).

Treatment of Hypotension and Shock

Management of hypotension and shock is difficult. Patients often are modestly dehydrated from heat, fever, anorexia, vomiting, and diarrhea, in any combination. There is extensive loss of intravascular volume through hemorrhage and increased vascular permeability.²⁷² Nevertheless, these patients often respond poorly to fluid infusions and readily develop pulmonary edema, possibly from myocardial impairment and increased pulmonary vascular permeability. Asanguineous fluids (either colloid or crystalloid solutions) should be given, with caution. Although it has never been evaluated critically for VHFs, dopamine might be the agent of choice for patients with shock who are unresponsive to fluid replacement. Alpha-adrenergic vasoconstricting agents

have not been clinically helpful except when emergent intervention to treat profound hypotension is necessary. Vasodilators have not been systematically evaluated. Pharmacological doses of corticosteroids (eg, methylprednisolone 30 mg/kg) provide another possible, but untested, therapeutic modality in treating shock.

Isolation and Containment

Patients with VHF syndrome (with the exception of dengue and classical hantavirus disease) generally have significant quantities of virus in their blood, and perhaps in other secretions as well. Secondary infections among contacts and medical personnel not parenterally exposed are well documented. Thus, caution is needed when evaluating and treating patients with suspected VHF syndrome. Overreaction by medical personnel is inappropriate and detrimental to both the patient and staff, but it is prudent to provide isolation measures as rigorous as feasible.²⁷³ At a minimum, isolation measures should include the following:

- Restricted access to the patient and use of stringent barrier nursing including mask, gown, glove, and needle precautions.
- Proper hazard labeling of all specimens submitted to the clinical laboratory with notification of appropriate clinical personnel.
- Proper disposal of all material within the isolation room by autoclaving or liberal disinfection of contaminated materials using such disinfectants as hypochlorite or phenols.

For more intensive care, however, increased precautions are recommended. Members of the patient-care team should be limited to a small number of selected, trained individuals, and special care should be directed toward eliminating all parenteral exposures. Use of endoscopy, respirators, arterial catheters, routine blood sampling, and extensive laboratory analysis increases opportunities for aerosol dissemination of infectious blood and body fluids. For medical personnel, wearing flexible plastic hoods equipped with battery-powered blowers provides excellent protection of the mucous membranes and airways.

PREVENTION AND CONTROL

Active Vaccination

With the possible exception of yellow fever, outbreaks of VHF have been relatively infrequent, small in size compared to other infectious diseases, and confined largely to remote geographic locales; quarantine of sick patients has been effective in controlling epidemics. In the past, this small global market has generated little commercial interest for developing VHF vaccines. However, the increased concern about the potential of these viruses as biological weapons and the recent attention drawn to outbreaks of emerging and reemerging viruses, such as the 2004–2005 epidemic of Marburg HF in Angola, has dramatically changed perspectives on the need for VHF vaccines.

The only established and licensed virus-specific vaccine available against any of the HF viruses is the yellow fever live attenuated 17D vaccine, which is mandatory for travelers to endemic areas of Africa and South America (Table 13-2).²⁷⁴ For prophylaxis against AHF virus, a live attenuated Junin vaccine strain (Candid #1) was developed at USAMRIID²⁷⁵ as part of an international cooperative project (USAMRIID-Pan American Health Organization) and is available as an investigational new drug (IND). Candid #1 was proven to be effective in phase III studies in Argentina,²⁷⁶ and plans are proceeding to obtain a new drug license. Candid #1 elicits high levels of protective

antibodies lasting 9 years in approximately 90% of the people vaccinated with a single dose. This vaccine also protects against Bolivian HF in experimentally infected primates. Two IND vaccines were developed against RVF: a formalin-inactivated vaccine that requires three boosters, which has been in use for 20 years, and a live attenuated RVF viral strain (MP-12). The inactivated vaccine has been administered to laboratory workers and appears to be safe and efficacious, but the ability to produce this vaccine in the United States no longer exists.²⁷⁷

For the hantaviruses, five commercially available vaccines are being produced in China,²⁷⁸ but these vaccines are not generally considered acceptable by United States standards. Another USAMRIID product, a genetically engineered vaccinia construct expressing hantaviral structural proteins, is in phase II safety testing in US volunteers. A formalin-inactivated Kyasanur forest disease vaccine was protective in field trials in India.²⁷⁹ For dengue, many live attenuated strains of all four serotypes are entering phase II efficacy testing. However, none of the vaccines in phase I or II IND status will be available as licensed products soon.

For the remaining VHF agents, availability of effective vaccines is more distant but possible. As with the VHFs noted above, early attempts to develop vaccines against these viruses were based on classical approaches directed primarily at using inactivated

whole virion preparations as vaccines.^{280,281} Results from these studies were inconsistent and in general were unsuccessful. Recent VHF vaccine development

has been concentrated on various recombinant vectors for expression of VHF-encoded proteins in various combinations to induce protective immunity, and

TABLE 13-2

PREVENTION AND CONTROL OF VIRAL HEMORRHAGIC FEVERS IN HUMANS

Virus Family Genus	Disease	Preventive Vaccine	Treatment
Arenaviridae			
<i>Arenavirus</i>	Lassa fever	None	Supportive, Ribavirin ⁵
	Argentine HF	IND ^{1,2}	Supportive, Ribavirin, ^{6,7} immune plasma ⁸
	Bolivian HF	None [*]	Supportive, Ribavirin, ⁹ immune plasma ⁹
	Brazilian HF	None	Supportive, Ribavirin ¹⁰
	Venezuelan HF	None	Supportive, Ribavirin?
Bunyaviridae			
<i>Nairovirus</i>	Crimean-Congo HF	None	Supportive, Ribavirin ¹¹⁻¹³
<i>Phlebovirus</i>	Rift Valley fever	IND ³	Supportive, Ribavirin ¹⁴
<i>Hantavirus</i>	HFRS	None [†]	Supportive, Ribavirin ^{14,15}
Filoviridae			
<i>Ebolavirus</i>	Ebola HF	None	Supportive, rNAPc2 ^{16‡}
<i>Marburgvirus</i>	Marburg HF	None	Supportive
Flaviviridae			
<i>Flavivirus</i>	Dengue HF	None	Supportive
	Yellow fever	Licensed ⁴	Supportive
	Omsk HF	None	Supportive
	Kyasanur forest disease	None	Supportive

*Junin Candid #1 vaccine protects nonhuman primates against Bolivian HF.

[†]Several vaccines are commercially available in China.

[‡]A treatment in this case may have some utility but the value is untested and unknown.

HF: hemorrhagic fever

HFRS: hemorrhagic fever with renal syndrome

IND: investigational new drug

Data sources: (1) McKee KT Jr, Barrera-Oro JG, Kuehne AI, Spisso JA, Mahlandt BG. Candid No. 1 Argentine hemorrhagic fever vaccine protects against lethal Junin virus challenge in rhesus macaques. *Intervirology*. 1992;34:154-163. (2) Maiztegui JI, McKee KT Jr, Barrera-Oro JG, et al. Protective efficacy of a live attenuated vaccine against Argentine hemorrhagic fever. AHF Study Group. *J Infect Dis*. 1998;177:277-283. (3) Pittman PR, Liu CT, Cannon TL, et al. Immunogenicity of an inactivated Rift Valley fever vaccine in humans. *Vaccine*. 1999;18:181-189. (4) Monath TP. Yellow fever vaccine. *Expert Rev Vaccines*. 2005;4:553-574. (5) McCormick JB, King IJ, Webb PA, et al. Lassa fever. Effective therapy with ribavirin. *N Engl J Med*. 1986;314:20-26. (6) Enria DA, Briggiler AM, Levis S, Vallejos D, Maiztegui JI, Canonico PG. Tolerance and antiviral effect of ribavirin in patients with Argentine hemorrhagic fever. *Antiviral Res*. 1987;7:353-359. (7) McKee KT Jr, Huggins JW, Trahan CJ, Mahlandt BG. Ribavirin prophylaxis and therapy for experimental Argentine hemorrhagic fever. *Antimicrob Agents Chemother*. 1988;32:1304-1309. (8) Enria DA, Briggiler AM, Fernandez NJ, Levis SC, Maiztegui JI. Importance of dose of neutralizing antibodies in treatment of Argentine hemorrhagic fever with immune plasma. *Lancet*. 1984;2:255-256. (9) Kilgore PE, Ksiazek TG, Rollin PE, et al. Treatment of Bolivian hemorrhagic fever with intravenous ribavirin. *Clin Infect Dis*. 1997;24:718-722. (10) Barry M, Russi M, Armstrong L, et al. Brief report: treatment of a laboratory-acquired Sabia virus infection. *N Engl J Med*. 1995;333:294-296. (11) Ergonul O, Celikbas A, Dokuzoguz B, Eren S, Baykam N, Esener H. Characteristics of patients with Crimean-Congo hemorrhagic fever in a recent outbreak in Turkey and impact of oral ribavirin therapy. *Clin Infect Dis*. 2004;39:284-287. (12) Fisher-Hoch SP, Khan JA, Rehman S, Mirza S, Khurshid M, McCormick JB. Crimean-Congo hemorrhagic fever treated with oral ribavirin. *Lancet*. 1995;346:472-475. (13) Mardani M, Jahromi MK, Naieni KH, Zeinali M. The efficacy of oral ribavirin in the treatment of Crimean-Congo hemorrhagic fever in Iran. *Clin Infect Dis*. 2003;36:1613-1618. (14) Huggins JW. Prospects for treatment of viral hemorrhagic fevers with ribavirin, a broad-spectrum antiviral drug. *Rev Infect Dis*. 1989;11(suppl 4):S750-S761. (15) Huggins JW, Hsiang CM, Cosgriff TM, et al. Prospective, double-blind, concurrent, placebo-controlled clinical trial of intravenous ribavirin therapy of hemorrhagic fever with renal syndrome. *J Infect Dis*. 1991;164:1119-1127. (16) Geisbert TW, Hensley LE, Jahrling PB, et al. Treatment of Ebola virus infection with a recombinant inhibitor of factor VIIa/tissue factor: a study in rhesus monkeys. *Lancet*. 2003;362:1953-1958.

tested for protective efficacy in animal models of VHF. Vaccination with recombinant vaccinia viruses expressing Lassa viral proteins successfully protected a majority of cynomolgus and rhesus monkeys from lethal Lassa fever.^{177,282} However, a similar strategy using the recombinant vaccinia virus platform as a vaccine for Ebola virus failed to confer any protection to nonhuman primates against lethal Ebola HF.²⁴⁴ One especially promising strategy has been the use of adenovirus vectors expressing Ebola GP and/or NP genes to protect monkeys against lethal Ebola challenge.²⁸³⁻²⁸⁵ This platform should be readily adaptable to other HF viruses, and a multivalent VHF vaccine is a plausible possibility. An alternative presentation strategy uses an attenuated vesicular stomatitis virus vector expressing the HF viral glycoprotein of interest. This strategy has successfully protected monkeys against lethal Ebola challenge,²⁸⁶ Marburg challenge,²⁸⁶ and Lassa challenges.²⁸⁷ Other vaccination strategies are under investigation, including virus-like particles^{288,289} and alphavirus replicons.^{244,290} One technical obstacle to the development of a multivalent VHF vaccine is the potential for prior immunity to the vector, either through natural exposure or prior vaccination, to inhibit immunogenicity.

Postexposure Vaccination

Efforts to develop preventive vaccines against the HF viruses, particularly Ebola, Marburg, and Lassa viruses, have been the most encouraging. Even more encouraging is a result from a recent study in rhesus monkeys showing that the vaccine system based on recombinant vesicular stomatitis virus may not only have utility as a potent preventive vaccine but may also have potential as a postexposure modality.²⁹¹ Recombinant vesicular stomatitis virus vectors expressing the Marburg virus glycoprotein were administered to five macaques 20 to 30 minutes after a high-dose lethal injection of homologous Marburg virus. Three animals served as Marburg-positive controls and received nonspecific vectors. All five rhesus monkeys that were treated with the specific Marburg vectors as a postexposure treatment survived a high-dose lethal challenge of Marburg, but all of the control animals developed fulminant disease and died from the Marburg challenge. These results clearly warrant further investigation and potentially provide a new paradigm for treating HF viral infections.

Specific Antiviral Therapy

No antiviral drugs are approved by the US Food and Drug Administration for treating the VHFs. Treatment is primarily by supportive management

and palliative care with particular attention given to maintenance of hydration, circulatory volume, blood pressure, and the provision of supplemental oxygen. There is a critical need for the development of effective therapies to respond to outbreaks of VHF in Africa and South America and to counter potential acts of bioterrorism. In addition, the recent death of a Russian scientist after an accidental exposure to Ebola virus²⁹² emphasizes the need for medical countermeasures for postexposure prophylaxis. Considering the aggressive nature of VHF infections, in particular the rapid and overwhelming viral burdens, early diagnosis plays a significant role in determining the success of any intervention strategy.

Development of effective therapies has been slow for many reasons, including little commercial interest and the need for special containment facilities for safe research. In addition, development of antivirals has been problematic because of the rapid and tremendous increase in viral loads during the acute phase of illness. For example, viremia during the acute phase of Ebola infection of humans or nonhuman primates typically exceeds 6.5 log₁₀ plaque-forming units (pfu)/mL of sera,²⁵ and in nonhuman primates viremia can go from less than 2.0 log₁₀ pfu/mL to over 5.0 log₁₀ pfu/mL in 24 hours.¹¹⁴ Thus, a 50% inhibition of virus load may be insignificant in controlling the infection. Additionally, nonhuman primate models indicate that compounds that significantly inhibit Ebola replication in vitro or in rodents²⁹³ may have little efficacy when used in monkeys.²⁹⁴

Ribavirin, a nonimmunosuppressive nucleoside analogue with broad antiviral properties,²⁹⁵ is of proven value for some VHF agents. Ribavirin was shown to reduce mortality from Lassa fever in high-risk patients,²⁹⁶ and it presumably decreases morbidity in all patients with Lassa fever, for whom current recommendations are to treat initially with ribavirin 30 mg/kg, administered intravenously, followed by 15 mg/kg every 6 hours for 4 days, and then 7.5 mg/kg every 8 hours for an additional 6 days.²⁷³ Treatment is most effective if begun within 7 days of onset; lower intravenous doses or oral administration of 2 g followed by 1 g per day for 10 days also may be useful. Although oral ribavirin is approved by the US Food and Drug Administration for treating chronic hepatitis C virus infection in combination with IFN- α , intravenous ribavirin is of limited availability in the United States. Oral ribavirin is manufactured by ICN Pharmaceuticals Inc (Costa Mesa, Calif) for compassionate use under an IND application.

The primary adverse effects caused by ribavirin have been anemia and hyperbilirubinemia related to a mild hemolysis and reversible block of erythropoiesis. The anemia did not require transfusions or cessation

of therapy in the published Sierra Leone study²⁹⁶ or in subsequent unpublished limited trials in West Africa. Ribavirin, which is contraindicated in pregnant women, is classified as a pregnancy category X drug.²⁹⁷ However, in VHF cases of unknown etiology or secondary to an *Arenavirus* or RVF virus, the benefits of treatment are likely to outweigh any fetal risk. Safety of oral or intravenous ribavirin in infants and children has not been established; aerosolized ribavirin has been approved by the Food and Drug Administration to treat respiratory syncytial virus infection in children.

A similar dose of ribavirin initiated within 4 days of disease is efficacious in patients with HFRS.^{298,299} In Argentina, ribavirin can reduce virological parameters of Junin virus infection,³⁰⁰ and is now used routinely as an adjunct to immune plasma. Unfortunately, ribavirin does not penetrate the brain and is expected to protect only against the visceral, and not the neurological, phase of Junin infection.³⁰¹

Small studies investigating the use of ribavirin in treating Bolivian HF and CCHF have been promising,^{155,302-304} as have preclinical studies for RVF.²⁹⁸ Conversely, ribavirin is ineffective against both the filoviruses and the flaviviruses, although a recent study with experimental animals suggests that ribavirin may have some therapeutic utility against yellow fever.³⁰⁵ Ribavirin is approved for use in treating VHF caused by arenaviruses and bunyaviruses, but not filoviruses, under the compassionate use provisions for INDs. Ribavirin was successfully used to treat a laboratory-acquired Sabia virus infection.³⁰⁶

Different preparations of type I IFNs were used in many studies to determine their utility in treating VHFs, with little success.³⁰⁷⁻³⁰⁹ At the moment, the type I IFNs appear to have little role in therapy, with the possible exception of RVF, in which fatal HF has been associated with low IFN responses in laboratory animals.³¹⁰ Exogenous IFN- γ was also shown to hold promise for treating RVF infections³¹¹; its role in treating other VHFs is unknown.

Several anti-gene strategies, including approaches based on phosphorodiamidate morpholino oligomers and small interfering RNAs, have been successfully used to protect rodents against Ebola HF³¹²⁻³¹⁴; however, as mentioned previously, further interest in these strategies is critically dependent on demonstration of postexposure protection in the more stringent nonhuman primate models.

Immunoprophylaxis and Immunotherapy

Passive immunotherapy has been attempted for treating the diseases caused by VHFs owing to the limited availability of effective antiviral drugs. Stud-

ies and case reports describing successes and clinical utility^{149,315-322} are frequently tempered by more systematic studies, where efficacy is less obvious or of no benefit.^{296,323,324} In the case of dengue virus, passively treating rhesus monkeys with antibody to dengue type 2 virus was associated with enhanced dengue type 2 replication.³²⁵ For all HF viruses, the benefit of passive treatment seems to be correlated with the concentration of neutralizing antibodies, which are readily induced by some, but not all, of these viruses.^{322,326-328}

Argentine HF responds to antibody therapy with two or more units of convalescent plasma that contain adequate amounts of neutralizing antibody (or an equivalent amount of immune globulin), provided that treatment is initiated within 8 days of onset.³¹⁶ Antibody therapy is also beneficial for treating Bolivian HF.³⁰³ Efficacy of immune plasma for treating Lassa fever³²⁷ and CCHF³²⁸ is limited by low neutralizing antibody titers and the consequent need for careful donor selection.

In the future, passive treatment strategies with recombinant human monoclonal antibodies may have utility against the VHF agents given the potential benefit of passive treatment described in many studies.^{316,321,322} In HFRS, a passive treatment approach is contraindicated for therapy because an active immune response is usually already evolving in most patients when they are first recognized, although plasma containing neutralizing antibodies has been used empirically in prophylaxis of high-risk exposures.

Modulation of the Host Immune Response

In addition to therapies that are directed toward inhibiting viral replication, strategies to modulate the host response or mitigate the effects of disease may have some utility and should be actively pursued. Two patients infected with Marburg virus in 1975 were given vigorous supportive treatment and prophylactic heparin.¹³⁵ This apparent success inspired the use of heparin to treat one of the Ebola patients in the original 1976 outbreak in Zaire²⁴³; unfortunately, this was unsuccessful. An alternative strategy for Ebola is inhibition of the procoagulant tissue factor pathway. The basis for this speculation is that Ebola virus infection induces overexpression of tissue factor in primate monocytes and macrophages.²³⁹ Based on these data, it was postulated that blocking factor VIIa/tissue factor might be beneficial after Ebola infection.²³⁰ In a preliminary study, nine Ebola-infected monkeys were treated with a protein, recombinant nematode anticoagulant protein c2 (rNAPc2), which prevents blood clotting, and three Ebola-infected monkeys were given a placebo control.²³⁰ Three of the nine treated animals survived, but all three that

were given the placebo control died. In addition, there was a significant delay in death in treated animals that succumbed to the Ebola challenge. Because Ebola infection is nearly 100% fatal in monkeys and kills up to 90% of infected humans, a 33% survival rate for one of the most virulent diseases known is a significant step forward in beginning to develop

ways to combat such pathogens. Other study results include the observation that protection of animals was associated with antithrombotic and antiinflammatory effects of the drug, suggesting that strategies that modulate the proinflammatory response may have some therapeutic utility and warrant further investigation.

SUMMARY

During the past decade, extensive coverage has been allocated, in both the popular press and scientific media, to agents causing VHF. Additional information on the VHFs is contained in recent review articles and book chapters.^{53,329-332} Some of these viruses may be exploitable as agents of terrorism because they are highly infectious, especially by aerosol, and produce high morbidity and mortality, especially in populations with no prior exposure or herd immunity. Although these viruses vary in their intrinsic attributes and

potential use as weapons, all can be introduced into naive populations via natural processes, with fearsome consequences. Increased concern about such natural or unnatural introductions has driven increased investment in basic research and construction of a network of biocontainment laboratories. The dividend will be a more fundamental understanding of the disease processes associated with these infections and identification of potential targets for antiviral drugs, vaccines, and generic countermeasures.

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Chapter 14

STAPHYLOCOCCAL ENTEROTOXIN B AND RELATED TOXINS

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INTRODUCTION

DESCRIPTION OF THE AGENT

PATHOGENESIS

CLINICAL DISEASE

- Fever
- Respiratory Symptoms
- Headache
- Nausea and Vomiting
- Other Signs and Symptoms

DETECTION AND DIAGNOSIS

MEDICAL MANAGEMENT

IMMUNOTHERAPY

VACCINES

SUMMARY

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INTRODUCTION

The gram-positive bacteria *Streptococcus pyogenes* and *Staphylococcus aureus* extensively colonize the human population and are frequent opportunistic pathogens. These bacteria secrete a variety of enzymatic and nonenzymatic virulence factors that are responsible for many disease symptoms. Among these factors, staphylococcal enterotoxins (SEs), toxic shock syndrome toxin (TSST-1), and streptococcal pyrogenic exotoxins of *S pyogenes* share a common three-dimensional protein fold characteristic of the bacterial products called "superantigens" because of their profound effects upon the immune system. Most strains of *S aureus* and *S pyogenes* examined harbor genes for superantigens and are likely to produce at least one of these products. The staphylococcal enterotoxins are most frequently associated with food poisoning, yet not all superantigens are enterotoxins, and more severe physiological consequences, such as a life-threatening toxic shock syndrome, 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.¹ 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.²

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. US scientists had completed studies that clearly demonstrated the effectiveness of SEB as a biological weapon before the ban on offensive toxin weapons announced by President Nixon in February 1970 (3 months after replicating agent weapons were banned). SEB was exceptionally suitable as a biological agent because its effect was produced with much less material than was necessary with synthetic chemicals, and it presumably had an exceptional "safety ratio" (calculated by dividing the effective dose for incapacitation by the dose producing lethality). However, the safety ratio is misleading because the coadministration of SEB or related toxins with replicating pathogens may profoundly lower the lethal dose. Available countermeasures and diagnostics have focused on SEB because of its historical significance in past biowarfare efforts; however, SEB represents many (perhaps hundreds) of related biologically active superantigens that are readily isolated and manipulated by recombinant DNA techniques. All of these superantigens are presumed to have a similar mode of biological action, but very little data are available for confirmation.

DESCRIPTION OF THE AGENT

An examination of genes encoding superantigens of *S aureus* and *S pyogenes* indicates a common origin or perhaps an exchange of genetic elements between bacterial species. The great diversity of superantigens and the highly mobile nature of their genetic elements also suggest an accelerated rate of evolution. Staphylococcal and streptococcal strains that colonize domestic animals are potential genetic reservoirs for new toxin genes,³ and the transfer of these sequences may contribute to hybrid polypeptides. However, the many similarities among severe diseases caused by *S aureus* and *S pyogenes* superantigens⁴ imply a common mechanism of pathology. Amino acid sequence comparisons indicate that superantigens can be loosely compiled into three major subgroups and numerous sequence variations⁵; whereas genetic analysis shows that they are all likely derived from common ancestral genes. Despite significant sequence divergence, with similarities as low as 14%, overall protein folds are similar among staphylococcal and streptococcal superantigens. The toxin genes have evolved by strong selective pressures to maintain receptor-binding surfaces by preserving three-dimensional protein structure. The

contact surfaces with human leukocyte antigen DR (HLA-DR) receptors involve variations of conserved structural elements,^{6,7} 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 superantigens⁸ 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 identification of samples.

Molecular details of the biological actions of bacterial superantigens are well established. Superantigens target cells mediating innate and adaptive immunity, resulting in an intense activation and subsequent pathology associated with aberrant host immune responses. Class II molecules of the major histocompatibility complex (MHC) are the primary receptors, and the MHC-bound superantigen in turn stimulates T cells. Most superantigens share a common mode for binding class II MHC molecules, with additional stabilizing interactions that are unique to each one.⁹

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 T-cell antigen receptors and class II MHC molecules on antigen-presenting cells (Figure 14-1). Superantigens also cross-link T-cell antigen receptors and class II MHC molecules, mimicking the CD4 molecule,¹⁰ and hence stimulate large numbers of T cells. In addition, each superantigen preferentially stimulates T cells bearing distinct subsets of antigen receptors, predominantly dictated by the specific V β chain. An intense and

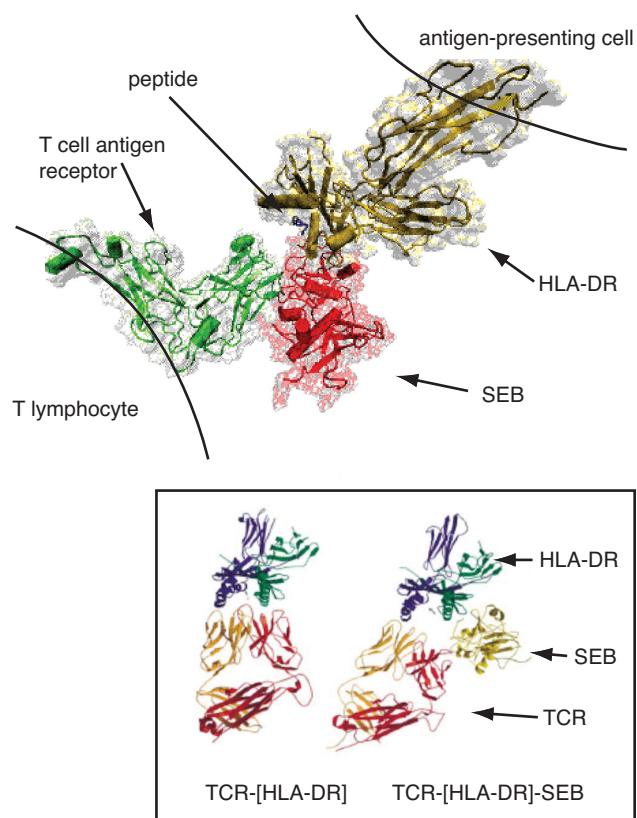


Fig. 14-1. Molecular model of receptor binding. Staphylococcal enterotoxins and other bacterial superantigens target the multireceptor communication between T cells and antigen-presenting cells that is fundamental to initiating pathogen-specific immune clearance. The superantigen inserts itself between the antigen receptor of T cells and the class II major histocompatibility complex 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

rapid release of cytokines such as interferon- γ , interleukin-6 and tumor necrosis factor- α is responsible for the systemic effects of the toxins.¹¹ In addition to direct T-cell activation, the gastrointestinal illness especially prominent after ingestion of staphylococcal enterotoxins is also associated with histamine and leukotriene release from mast cells.¹² Furthermore, the CD44 molecule reportedly provides protection from liver damage in mice caused by SEB exposure through a mechanism linked to activation-induced apoptosis of immune cells.¹³

Individuals within the human population 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, DQ, and DP isotypes and distinct alleles of class II MHC molecules, observed by differences in T-cell responses in vitro. In addition, primates, including humans, are most sensitive to superantigens compared to other mammals.¹⁴ Lethal or incapacitating doses of toxin may be lowered by coexposure to endotoxin from gram-negative bacteria¹¹ or hepatotoxins,¹⁵ or by infection with replicating agents.²

Rodents and other domestic animals infected with strains that produce TSST-1 and SE^{16,17} are potential environmental reservoirs. Both ovine- and-bovine specific staphylococcal toxins, which are associated with mastitis, are almost identical to TSST-1 in amino acid sequence.¹⁸ Toxicogenic strains are frequent or universal in both clinical and nonclinical isolates of *S aureus* and *S pyogenes*, and these strains contribute significantly to several diseases. Approximately 50% of nonmenstrual toxic shock syndrome (TSS) cases are linked to TSST-1, while the remaining cases are attributable to SE, with SEB predominating.¹⁹ Kawasaki's syndrome and some forms of arthritis are loosely associated with organisms producing streptococcal pyrogenic exotoxins (SPEs), SEA, and TSST-1.²⁰ In addition, streptococcal pneumonia with accompanying TSS-like symptoms is caused by SPE-producing bacteria.²¹

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 is present in strains of *S aureus*.²² Because little evidence of genetic drift exists, it has been hypothesized that the majority of staphylococcal and streptococcal TSS-like bacterial isolates have each descended from single clones.²³ Production of many SEs is dependent on the phase of cell-growth cycle, environmental pH, and glucose concentration. Transcriptional control of TSST-1, SEB, SEC, and SED is mediated through the accessory gene regulator (*agr*) locus,²⁴ whereas SEA expression appears to be independent of *agr*. Strains that are *agr*-negative are generally low toxin producers.

However, there are also considerable differences in production levels among agr-positive isolates. In addition, a feedback-mediated regulatory mechanism for increasing expression of SEB and TSST-1 and suppressing all other exotoxins has been demonstrated.²⁵

At the cellular level, the interaction of superantigens with receptors on antigen-presenting cells and T cells leads to intracellular signaling.²⁶ High concentrations of SEB elicit phosphatidyl inositol production and activation of protein kinase C and protein tyrosine kinase pathways,^{26–28} similar to mitogenic activation of T cells. SEs also activate transcription factors NF- κ B and AP-1, resulting in the expression of proinflammatory cytokines, chemokines, and adhesion molecules. Both interleukin-1 and tumor necrosis factor- α can directly activate the transcription factor NF- κ B in many cell types, including epithelial cells and endothelial cells, perpetuating the inflammatory response. Another mediator, interferon- γ , produced by activated T cells and natural killer cells, synergizes with tumor necrosis factor- α and interleukin-1 to enhance immune reactions and promote tissue injury. The substances induced directly by SEB and other superantigens—chemokines, interleukin-8, monocyte chemoattractant protein-1, macrophage inflammatory protein-1 α , and macrophage inflammatory protein-1 β —can selectively chemoattract and activate leukocytes. 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; tissue factor; and chemokines from 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.

Given the complex pathophysiology of toxic shock, the understanding of the cellular receptors and signaling pathways used by staphylococcal superantigens,

and the biological mediators they induce, 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^{29–32}; (b) inhibition of signal transduction pathways used by SEs²⁶; (c) inhibition of cytokine and chemokine production^{33,34}; and (d) inhibition of the downstream signaling pathways used by proinflammatory cytokines and chemokines.

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. Although several clinical trials of treatment of sepsis with high-dose corticosteroids were unsuccessful, a multicenter clinical trial using lower doses of corticosteroids for longer periods reduced the mortality rate of septic shock.³⁵ A newer intervention targeting the coagulation pathway by activated protein C improved the survival of septic patients with high APACHE (Acute Physiology and Chronic Health Evaluation, a system for classifying patients in the intensive care unit) score.³⁶ Because coagulation and endothelial dysfunction are important facets of SEB-induced shock, activated protein C may also be useful in treating TSS.

Limited therapeutics for treating superantigen-induced toxic shock are currently available. Intravenous immune globulin was effective as a treatment in humans after the onset of TSS. Antibody-based therapy targeting direct neutralization of SEB or other superantigens represents another form of therapeutics, most suitable during the early stages of exposure before cell activation and the release of proinflammatory cytokines. Because some neutralizing antibodies cross-react among different superantigens,⁸ 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.^{37,38}

PATHOGENESIS

Rhesus macaques (*Macaca mulatta*) have been used extensively as a model for lethal disease caused by inhaled SEB. Rabbits, endotoxin-primed mice, and additional animal models have been developed. Because SEB and related toxins primarily affect primates, the following unpublished rhesus monkey data are highly relevant for understanding potential human pathology. Young and mature adult male and female rhesus

monkeys developed signs of SEB intoxication³⁹ after being exposed to a lethal dose of aerosolized SEB for 10 minutes in a modified Henderson head-only aerosol exposure chamber.⁴⁰ These animals demonstrated no detectable anti-SEB antibody before exposure. After inhalation exposure, microscopic lymphoproliferation of T-cell-dependent areas of the lymphoid system, consistent with the potent stimulatory effect of SEB

on the rhesus monkey immune system, was apparent. Immunohistochemical analysis, using anti-CD3 antibody, of the large lymphocytes present in the pulmonary vasculature of the monkeys identified these lymphocytes as T cells.⁴¹

Generally, the SEB-intoxicated rhesus monkeys developed gastrointestinal distress within 24 hours post-exposure. Clinical signs were mastication, anorexia, emesis, and diarrhea. After mild, brief, self-limiting gastrointestinal signs, the monkeys had a variable period of up to 40 hours of clinical improvement. At approximately 48 hours postexposure, the monkeys generally had an abrupt onset of rapidly progressive lethargy, dyspnea, and facial pallor, culminating in death or euthanasia within 4 hours of onset.

At necropsy, most of the monkeys had similar gross pulmonary lesions. The lungs were diffusely heavy and wet, with multifocal petechial hemorrhages and areas of atelectasis. Clear serous-to-white frothy fluid often drained freely from the laryngeal orifice. The small and large intestines frequently had petechial hemorrhages and mucosal erosions. Typically, the monkeys had mildly swollen lymph nodes, with moist and bulging cut surfaces.

Most of the monkeys also had similar microscopic pulmonary lesions. The most obvious lesion was marked multifocal to coalescing interstitial pulmonary edema involving multiple lung lobes. Peribronchovascular connective tissue spaces were distended by pale, homogeneous, eosinophilic, proteinaceous material (edema), variably accompanied by entrapped, beaded fibrillar strands (fibrin), extravasated erythrocytes, neutrophils, macrophages, and small and large lymphocytes. Perivascular lymphatics were generally distended by similar eosinophilic material and inflammatory cells. Most of the monkeys had intravascular circulating and margined neutrophils, monocytes, mononuclear phagocytes, and lymphocytes, including large lymphocytes with prominent nucleoli (lymphoblasts), some in mitosis (Figure 14-2). Extravascular extension of these cell types was interpreted as exocytosis/chemotaxis.

Loss of airway epithelium was inconsistent. Some monkeys had multifocal, asymmetric denudation of bronchial epithelium, with near total loss of bronchiolar epithelium. Former bronchioles were recognized only by their smooth muscle walls. Scant bronchial intraluminal exudate consisted of mucoid material, neutrophils, macrophages, and sloughed necrotic cells.

A common finding was multifocal alveolar flooding and acute purulent alveolitis. Alveolar septa were distended by congested alveolar capillaries. Alveolar spaces were filled with pale, homogeneous, eosinophilic material (edema), with deeper embedded

eosinophilic beaded fibrillar strands (fibrin), or with condensed, curvilinear, eosinophilic deposits hugging the alveolar septal contours (hyaline membranes). A variably severe cellular infiltrate of neutrophils, eosinophils, small lymphocytes, large lymphocytes (lymphoblasts), erythrocytes, and alveolar macrophages filled alveolar spaces. Replicate pulmonary microsections stained with phosphotungstic-acid-hematoxylin demonstrated alveolar fibrin deposition. Replicate microsections stained with Giemsa revealed scarce sparsely granulated connective-tissue mast cells.

In the upper respiratory tract, the tracheal and bronchial lamina propria was thickened by clear space or pale, homogeneous, eosinophilic material (edema), neutrophils, small and large lymphocytes, and (possibly preexisting) plasma cells. The edema and cellular infiltrate extended transtracheally into the

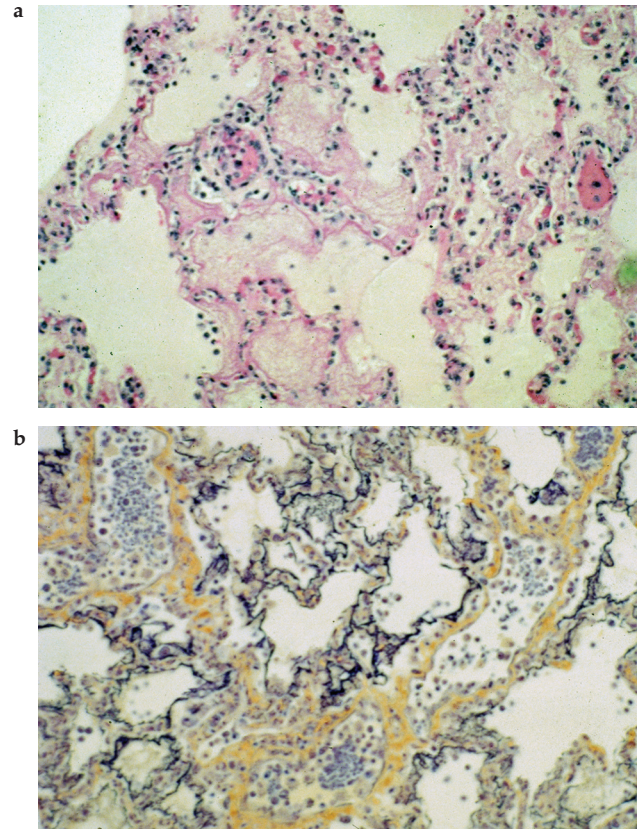


Fig. 14-2. Lung of a rhesus monkey that died from inhaled staphylococcal enterotoxin B. (a) Marked perivascular interstitial edema and focal loss of bronchial epithelium can be seen (hematoxylin-eosin stain, original magnification $\times 10$). (b) The intravascular mononuclear cells include lymphocytes, lymphoblasts, monocytes, and mononuclear phagocytes (hematoxylin-eosin stain, original magnification $\times 50$).

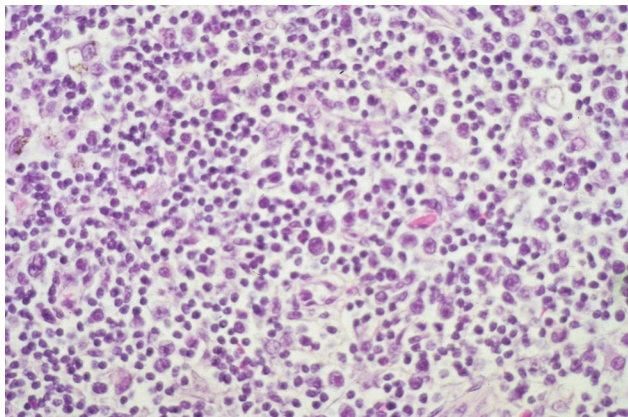


Fig. 14-3. Mediastinal lymph node of a rhesus monkey that died from inhaled staphylococcal enterotoxin B. Paracortical lymphoproliferation with lymphoblasts can be seen (hematoxylin-eosin stain, original magnification x 100).

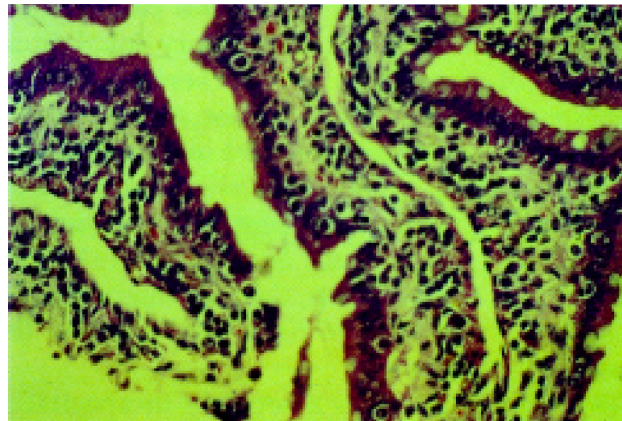


Fig. 14-4. Small intestine of a rhesus monkey that died from inhaled staphylococcal enterotoxin B. Intraepithelial lymphoblastic leukocytes can be seen (hematoxylin-eosin stain, original magnification x 100).

mediastinum, with moderate to marked mediastinal lymphangiectasia.

Lymphoid tissues of the respiratory tract had depletion of B-cell-dependent areas and hyperplasia of T-cell-dependent areas. The bronchus-associated lymphoid tissue in some of the monkeys had follicular lymphocytic depletion. Most of the mediastinal lymph nodes had subcapsular and medullary sinus edema, histiocytosis, and paracortical lymphoid hyperplasia, characterized by numerous closely packed small lymphocytes with interspersed macrophages bearing tingible bodies and large lymphocytes having prominent nucleoli (lymphoblasts) (Figure 14-3). There were scattered mitoses, including atypical mitoses. Cortical follicles had small solid centers or hypocellular, hyalinized (depleted) centers.

Microscopic changes in lymphoid tissues elsewhere in the body mirrored changes in the respiratory mucosal lymphoid tissue. Mesenteric, axillary, inguinal, and retropharyngeal lymph nodes had sinus edema and histiocytosis, paracortical lymphocytic and lymphoblastic hyperplasia, and unstimulated or depleted follicular centers. Also depleted were follicular germinal centers of gut-associated lymphoid tissue. Splenic T-cell-dependent periarteriolar sheath zones were

hypercellular, populated by a mix of small and large lymphocytes and macrophages, whereas B-cell-dependent follicular areas were not recognized. Several monkeys had marked diffuse depletion of cortical thymocytes, with a “starry sky” appearance attributed to the presence of numerous thymic macrophages bearing tingible bodies.

Many of the monkeys had a mild erosive enterocolitis, with slight, superficial, multifocal mucosal loss and with numerous lamina propria macrophages bearing engulfed cellular debris. Crypt enterocytes had a high nuclear-to-cytoplasmic ratio and numerous mitoses. The crypt epithelium had a conspicuous population of large mononuclear intraepithelial leukocytes interpreted as lymphoblasts (Figure 14-4). In the colon of some monkeys, there were many small crypt abscesses.

Generalized vascular changes in most of the monkeys were congestion, swollen endothelial cells with many large intravascular lymphocytes or lymphoblasts and inconsistent widening of perivascular connective tissue spaces (by edema). Hepatic lesions were portal infiltrates of lymphocytes, lymphoblasts, macrophages, and occasional neutrophils. The choroid plexus was slightly thickened by edema.

CLINICAL DISEASE

The clinical documentation of TSS provides perhaps the most comprehensive source of information on the pathology of superantigen (eg, SEB) exposure. To meet the strict Centers for Disease Control and Prevention criteria for TSS,⁴² negative blood (except for *S aureus* or *S pyogenes*), throat, or cerebrospinal fluid cultures, as well

as negative serologic tests for Rocky Mountain spotted fever, leptospirosis, and measles should be obtained. Although TSS disease symptoms are well established, characterized by a rapid drop in blood pressure, elevated temperature, and multiple organ failure, the respiratory route of exposure may involve some unique mechanisms.

The profound hypotension and desquamation of the palms and soles of the feet that are characteristic of TSS are not observed in exposure by inhalation, and respiratory involvement is rapid, unlike in other forms of TSS. Furthermore, the fever prominent after aerosol exposure is generally not observed in cases of SEB ingestion.

Documentation of an accidental laboratory inhalation exposure of nine laboratory workers to SEB best exemplifies the clinical disease, described as a severely incapacitating illness of rapid onset (3–4 hours) and modest acute duration (3–4 days).⁴³

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. Onset ranged 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 & Co, Indianapolis, Ind) 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.⁴⁴

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.^{45,46} Immunoassays can detect picogram quantities of toxins in environmental samples. Plasma concentrations of superantigens were measured in septic patients of an intensive care unit using an enzyme-linked immunosorbent assay.⁴⁷ In one study,⁴⁸ 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^{49,50} 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 by using polymerase chain reaction amplification and toxin gene-specific oligonucleotide primers. The results from both polymerase chain reaction 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. Supportive therapy in the nine mild accidental exposure cases described above 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. Diarrhea was not observed in human accidental exposure cases, but 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.

IMMUNOTHERAPY

Infusion of intravenous immunoglobulin has been successfully used^{51,52} 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.⁵³ Unpublished studies have documented the prophylactic and therapeutic value of human intravenous immunoglobulin in rhesus monkeys after inhalation of SEB, prescribed to the

presence of antibodies to SE and TSST-1 in commercial preparations of intravenous immunoglobulin and normal human sera. 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 < 5 min), active immunity should be considered as the best defense.

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 interdermal routes, have also shown promising results in animal 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,^{7,9,37,38} without altering the three-dimensional structure of the antigen. Though promising, these engineered vaccines are not yet licensed or available for general use.

SUMMARY

SEB is representative of a group of bacterial proteins that exerts 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.

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Chapter 15

RICIN

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INTRODUCTION

Ricin is a protein isolated from the seeds of the castor bean plant (*Ricinus communis*). Like abrin (from the seeds of the rosary pea, *Abrus precatorius*), ricin is a lectin and a member of a group of ribosome-inactivating proteins that block protein synthesis in eukaryotic ribosomes.¹

The castor bean is native to Africa, but it has been introduced and cultivated throughout the tropical and subtropical world. Although tolerant to a wide temperature range, it grows best in elevated year-round temperatures and rapidly succumbs to sub-freezing temperatures. However, it is often grown as an ornamental annual in temperate zones. The seeds are commercially cultivated in many regions of the world, predominantly in Brazil, Ecuador, Ethiopia, Haiti, India, and Thailand. The beans contain 35% to

55% by weight of fast-drying, nonyellowing oil used in the manufacture of lubricants, inks, varnishes, and dyes. After oil extraction, the remaining seed cake may be detoxified by heat treatment and used as an animal feed supplement. The seed hulls are similar to barnyard manure in their fertilizer value.

The toxicity of castor beans has been known since ancient times, and more than 750 cases of intoxication in humans have been described.² Although considerably less potent than botulinum neurotoxins and staphylococcal enterotoxins, ricin represents a significant potential biological weapon because of its stability and worldwide availability as a by-product of castor oil production. In addition, it has been associated with several terrorist actions and therefore may be a potential agent of bioterror.

HISTORY

R. communis was cultivated for centuries in ancient Egypt and Greece for the lubricating and laxative effects of its oil. In addition, both the oil and whole seeds have been used in various parts of the world for disease treatment as well as for malicious mischief and homicidal purposes.³ During World War I, the excellent lubricating properties of castor oil were utilized by the wartime aircraft industry. Shortages of castor oil during World War II resulted in US government subsidies for agricultural production of castor beans in the San Joaquin Valley of California. These subsidies persisted until the 1960s, when synthetic oils replaced castor oil in the aircraft industry. There is no commercial production of castor oil in the United States today.

The first toxinology work on ricin was performed by Hermann Stillmark at the Dorpat University in Estonia for his 1888 thesis.⁴ Stillmark determined that ricin was a protein and suggested the name. He purified ricin to a very high degree (although not completely to homogeneity) and found that it agglutinated erythrocytes and precipitated serum proteins.⁵ For years, these effects were considered to be the mechanism of action of ricin, although later work showed that the toxicity and agglutination effects were separable properties.

In 1891 Paul Ehrlich studied ricin and abrin in pioneering research that is now recognized as the foundation of immunology.⁵ Following the lead of Indian farmers who had known for centuries that calves could be protected from abrin poisoning by feeding them small amounts of *Abrus* seeds, Ehrlich vaccinated animals with small oral doses of castor beans. After protection was established, he continued vaccinating with subcutaneous injections of toxin. Experiments

with the serum of immune animals led him to discover that the immunity was specific, was associated with serum proteins, and could be transferred to the offspring through milk.

At the end of the 19th century, with the rising interest in bacterial toxins, interest in plant toxins waned. It wasn't until the mid-20th century, with the discovery that ricin inhibited protein synthesis and thus might be useful for treating cancer, that the scientific community "rediscovered" ricin. Olsnes and Pihl⁶ demonstrated that protein synthesis was strongly inhibited in a cell-free rabbit reticulocyte system, and suggested that the effects resulted from inhibited elongation of the nascent polypeptide chain. They also determined that ricin consisted of two dissimilar polypeptide subunits and that the A chain was responsible for the toxic action. Results from this laboratory over the next few years revealed the 60S ribosomal subunit as the enzymatic target and led to further characterization of the enzymatic action.⁷

More recently, the inhibitory action of ricin on protein synthesis in eukaryotic cells was investigated as a potential chemotherapeutic agent against some forms of cancer. The active subunit of ricin is specifically targeted to tumor cells by conjugation to tumor-specific antibodies. These chimeric toxins, called immunotoxins, have been tested against several forms of cancer, with promising results.⁸ However, side effects such as nonspecific hepatic toxicity and vascular leak syndrome (VLS) have been problematic and dose limiting. Recent work by Smallshaw and coworkers⁹ has demonstrated that the VLS activity of the toxin is mediated by a discrete sequence moiety separate

from the region related to protein synthesis inhibition. Specifically, mutations in a three-amino acid motif of the ricin A chain yielded an immunotoxin with significantly reduced VLS side effects with no loss of cytotoxicity. Testing in a mouse model demonstrated improved effectiveness, suggesting that ricin immunotoxins may yet have a place in the anticancer armamentarium.

Because of its potency, worldwide availability, and ease of production, the US Chemical Warfare Service began considering ricin as a potential biological warfare agent near the end of World War I. The research involved methods of adhering ricin to shrapnel and the production of effective aerosol clouds.¹⁰ However, the war ended before the evolution of weaponry based upon this research. During World War II, the Americans and British collaborated on the development of a ricin-containing bomb (the so-called “W bomb”). Although they were tested, these bombs were never used in battle. The United States unilaterally ended its offensive biological warfare program in 1969–1970; all offensive research and development were terminated, and remaining stocks of ricin munitions were destroyed in 1971–1972. The 1975 Convention on the Prohibition of the Development, Production and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction prohibited the development, production, and storage of any toxin for offensive purposes.

In addition to its coverage under the 1975 convention, ricin and one other toxin (saxitoxin) were also specifically included under the 1993 Chemical Weapons Convention, ratified by Congress in 1997. In the

United States, ricin and abrin are both included in the Centers for Disease Control and Prevention’s select agent list of toxins requiring certification for possession and transfer. The US intelligence community believes that ricin was included in the biological warfare programs of the Soviet Union, Iraq, and possibly other nations as well.

In recent years, ricin has drawn the interest of extremist groups. Such notoriety is likely driven by the ready availability of castor beans, ease of toxin extraction, coverage in the popular press, and popularization on the Internet. Several individuals have been arrested under the 1989 Biological Weapons Anti-Terrorism Act for possessing ricin. In the past few years alone, various major news organizations have reported the following stories:

- 2002: Ricin was discovered in the apartment of six terrorist suspects arrested in Manchester, England.
- 2003: An envelope containing a sealed container of ricin and a note threatening to contaminate water supplies was processed at a mail facility in Greenville, South Carolina.
- 2004: Traces of ricin were discovered in the mail room of the Dirksen Senate Office Building in Washington, DC.

While none of these events resulted in any known human intoxications, they clearly demonstrate that ricin is well known, available to and recognized by extremist groups, and should be seriously considered as a potential bioterrorist threat agent.

DESCRIPTION OF THE AGENT

Ricin is a 66-kd globular protein that typically makes up 1% to 5% of the dry weight of the castor bean, although the yield can be highly variable.¹¹ The toxic form is a heterodimer consisting of a 32-kd A chain connected to the 32-kd B chain through a single disulfide bond.¹² As such, it is a member of the type II family of ribosome-inactivating proteins (RIPs), which possess enhanced in-vivo toxicity because of the presence of the B chain that facilitates uptake by the cell. Type I RIPs lack the B chain, and cellular toxicity is much less; uptake depends on endocytosis. Both chains are glycoproteins containing multiple mannose residues on their surfaces; association of both chains is required for toxicity.

Purification and characterization is not difficult, and the crystal structure has been determined to .25 nm.¹³ Each chain is a globular protein, with the A chain tucked into a gap between two roughly spherical domains of the B chain. A lactose disaccharide moiety is

bound to each of these spherical domains. The disulfide bond links residue 259 of the A chain with residue 4 of the B chain. The crystal structure demonstrates a putative active cleft in the A chain, which is believed to be the site of enzymatic action. A functional lipase active site at the interface of the two subunits was recently identified.¹⁴ This site is thought to be important for intracellular A chain translocation and subsequent intracellular trafficking (see below). Recombinant A and B chains, as well as specific mutants, have been expressed and characterized in several expression systems including *Escherichia coli*.¹⁵⁻¹⁸

Toxicity

Ricin is recognized as one of the most exquisitely toxic plant-derived RIPs identified to date.¹⁹ However, considerable variation in potency exists among species.

For instance, on a mg/kg basis, potency varies over two orders of magnitude between species of domestic and laboratory animals; chickens and frogs are the least sensitive, and horses are the most sensitive.²⁰ Potency also varies greatly with route of administration. In laboratory mice, approximate median lethal dose values and time to death are, respectively, 5 µg/kg and 90 hours by intravenous injection, 22 µg/kg and 100 hours by intraperitoneal injection, and 24 µg/kg and 100 hours by subcutaneous injection. Ricin is extremely toxic by inhalation; median lethal dose estimates range from 3 to 15 µg/kg in rodents and primates (Table 15-1). In contrast, ricin is least potent by the oral route; median lethal dose estimates in mice are approximately 20 mg/kg. Low potency by the oral route likely reflects poor absorption and possibly partial degradation in the gut. Higher potency by other routes may be related to the ubiquitous nature of toxin receptors among cell types. In skin tests on mice, no dermal toxicity was observed at 50 µg/spot, suggesting poor dermal absorption of this large, highly charged protein.²¹

Pathogenesis

The mechanism of action of ricin is similar to that of other type II RIPs. The two-chain structure is key to cellular internalization and subsequent toxicity. The lectin properties of the B chain enable toxin binding to cell-surface carbohydrates, and the A chain possesses the enzymatic activity. Initial binding of the B chain to glycoside residues on glycoproteins and glycolip-

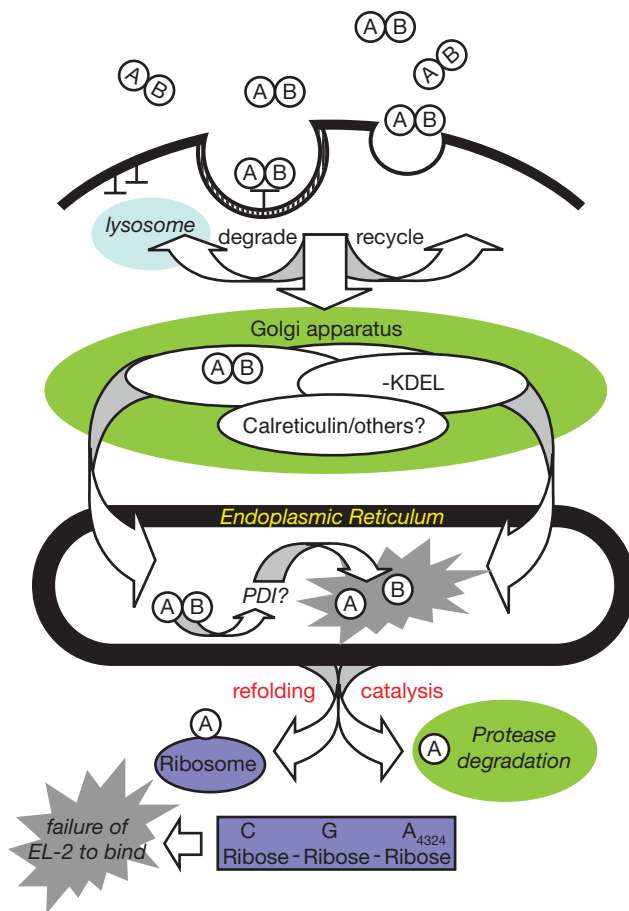


Fig. 15-1. Binding, internalization, and intracellular tracking of ricin leading to enzymatic action at the 60S ribosome. Endosomes transport ricin from the initial binding site to the Golgi apparatus (and may also traffic the internalized ricin back to the cell surface or to lysosomal degradation). Then, calreticulin and possibly other proteins are thought to chaperone the ricin from the Golgi apparatus to the endoplasmic reticulum (ER). At the ER, protein disulfide isomerase may reduce the disulfide bridge between the ricin subunits, facilitating unfolding and retrograde transport of the A chain through the ER lumen via a Sec61-mediated translocon. In the cytoplasm, the A chain can interact with the ribosome, which acts as a suicidal chaperone stimulating proper refolding and resumption of catalytic activity. The A chain cleaves one specific adenosine residue (A4324) near the 3' end of 28S ribosomal RNA, which blocks elongation factor-2 binding, thus inhibiting protein synthesis.

A: ricin A chain
 A4324: adenosine residue 4324
 B: ricin B chain
 EL-2: elongation factor 2
 -KDEL: amino acid sequence at the C-terminal of a soluble protein in the lumen of a membrane or a C-terminal Lys-Asp-Glu-Leu sequence
 PDI: protein disulfide isomerase
 Illustration: Courtesy of Chad Roy, Tulane National Primate Research Center, Covington, Louisiana.

TABLE 15-1
MEDIAN LETHAL DOSES FOR AEROSOLIZED RICIN IN VARIOUS ANIMAL SPECIES

Species	Strain	LD ₅₀ (µg/kg)
Mouse (<i>Mus musculus</i>)	BALB/c	11.2
	BXSB	2.8
	Swiss Webster	4.9
	CBA/J, C57/BL/6J,	
	L2H/HeJ	5.3
	A/J	8.2
Rat (<i>Rattus norvegicus</i>)	C3H/HeN	9.0
	Fisher 344	5.3
African green monkey (<i>Chlorocebus aethiops</i>)		5.8
Rhesus monkey (<i>Macaca mulatta</i>)		15.0

LD₅₀: medial lethal dose

ids triggers endocytic uptake of the toxin. Increased binding is observed in cell types rich in mannose receptors; dissociation of ricin from its binding sites is increased in the presence of lactose.²² There are a number of possible endocytic mechanisms for cell entry, some of which are independent of cell coat-binding protein (clathrin) action.²³ Trafficking of the toxin within the cell from the initial binding site to the Golgi apparatus occurs via endosomal transport and is seemingly regulated by intracellular calcium.²⁴ Early endosomes may also traffic the internalized ricin back to the cell surface or to lysosomal degradation (Figure 15-1). A Golgi-associated type II- α protein kinase also largely regulates toxin transport in specific cell types such as lymphocytes.²⁵ Association with the Golgi apparatus seems to be a requirement for further trafficking to the endoplasmic reticulum (ER).²⁶ Transport from the Golgi apparatus to the ER is thought to be in association with one or more chaperone proteins, most notably calreticulin.²⁷ Once delivered to the ER, protein

disulfide isomerase may reduce the disulfide bridge between the subunits, facilitating unfolding and retrograde transport of the A chain through the ER lumen via a Sec61-mediated translocon.²⁸ ER processing and transport to the cytosol is a critical step; only when the holotoxin is reduced by novel chaperones such as protein disulfide isomerase can subsequent ribosomal inactivation take place in the cytosol. As with related toxins, transport to the cytosol is the rate-limiting step during the decline in protein synthesis.²⁹ Once transported from the ER to the cytoplasm, the A chain can interact with the ribosome, which acts as a suicidal chaperone stimulating proper refolding and resumption of catalytic activity.²⁸ The Michaelis constant for enzymatic action at the ribosome is 0.1 $\mu\text{mol/L}$ and the enzymatic constant is 1,500/min. It cleaves one specific adenosine residue (A4324) near the 3' end of 28S ribosomal RNA. This targeted cleavage blocks elongation factor-2 binding, thus inhibiting protein synthesis.³⁰ The rate of ribosomal inactivation easily overwhelms repair mechanisms and kills the cell.

CLINICAL SYMPTOMS, SIGNS, AND PATHOLOGY

Animal studies indicate that clinical signs and pathological changes in ricin intoxication are largely route specific. Ingestion causes gastrointestinal symptoms including hemorrhage and necrosis of liver, spleen, and kidneys; intramuscular intoxication causes severe localized pain, muscle and regional lymph node necrosis, and moderate systemic symptoms; inhalation results in respiratory distress with airway and pulmonary lesions. Transient leukocytosis appears to be a constant feature in humans, whether intoxication is by injection or oral ingestion. Leukocyte counts 2- to 5-fold above normal are characteristic findings among cancer patients receiving ricin immunotoxin therapy, and also in the case of the Bulgarian dissident Georgi Markov during his agonizing death after a successful assassination attempt.³¹

Oral Intoxication

Ricin is less toxic by oral ingestion than by other routes, probably due to poor absorption of the toxin and possibly partial enzymatic degradation in the digestive tract. In animal models, a significant amount of orally administered ricin is found in the large intestine 24 hours postingestion with limited systemic uptake.³² Most cases of oral ingestion are related to ingestion of castor beans, and the severity of intoxication varies with the degree of mastication of the beans. Review of the literature reveals mostly nonfatal case reports of castor bean ingestion in the United States and a

few fatal case reports from abroad. A review of the American Association of Poison Control Center's Toxic Exposure Surveillance System from 1983 to 2002 notes no reported fatalities from ricin poisoning.³³

A recent review article³⁴ summarizes symptoms of substantial castor bean ingestion. The authors note oropharyngeal irritation, vomiting, abdominal pain, and diarrhea beginning within a few hours of ingestion. Local necrosis in the gastrointestinal tract may lead to hematemesis, hematochezia, and/or melena. The resultant loss of fluid and electrolytes may lead to hypotension, tachycardia, dehydration, and cyanosis. Significant fluid loss may lead to renal failure and hypovolemic shock. A portion of the toxin is absorbed through the gastrointestinal tract leading to systemic signs. In oral (and parenteral) intoxication, cells in the reticuloendothelial system, such as Kupffer cells and macrophages, are particularly susceptible, due to the mannose receptor present in macrophages.³⁵ The effect on these cells may lead to liver damage, which may persist for several days and may progress to liver failure at higher doses.

In 1985 Rauber and Heard² summarized the findings from their study of 751 cases of castor bean ingestion. There were 14 fatalities in this study, constituting a death rate of 1.9%—much lower than traditionally believed. Twelve of the 14 cases resulting in death occurred before 1930. Even with little or no effective supportive care, the death rate in symptomatic patients has been low—in the range of 6%. The reported number

of beans ingested by patients who died varied greatly. Of the two lethal cases involving oral intoxication documented since 1930, one involved a 24-year-old man who ate 15 to 20 beans, and the other involved a 15-year-old boy who ate 10 to 12 beans. All of the reported serious, or fatal, cases of castor bean ingestion have the same general clinical history: rapid (less than a few hours) onset of nausea, vomiting, and abdominal pain followed by diarrhea, hemorrhage from the anus, anuria, cramps, dilation of the pupils, fever, thirst, sore throat, headache, vascular collapse, and shock. Death occurred on the 3rd day or later. The most common autopsy findings in oral intoxication were multifocal ulcerations and hemorrhages of gastric and small-intestinal mucosa. Lymphoid necrosis in the mesenteric lymph nodes, gut-associated lymphoid tissue, and spleen were also present, as were Kupffer cell and liver necrosis, diffuse nephritis, and diffuse splenitis.

Injection

Intramuscular or subcutaneous injection of high doses of ricin in humans results in severe local lymphoid necrosis, gastrointestinal hemorrhage, liver necrosis, diffuse nephritis, and diffuse splenitis. Injection of ricin leads to necrosis at the injection site, which may predispose one to secondary infection.³⁶ A case report of a 20-year-old male who injected castor bean extract to commit suicide describes in detail the clinical course. This patient was admitted 36 hours after the injection with severe weakness, nausea, dizziness, headache, and pain in the chest, abdomen, and back. Examination revealed hypotension, anuria, metabolic acidosis, and hematochezia. He subsequently developed a bleeding diathesis, liver failure, and renal failure. Despite maximal treatment with vasopressors and treatment of the bleeding diathesis, he developed cardiac arrest and was unable to be resuscitated. Postmortem examination revealed hemorrhagic foci in the brain, myocardium, and the pleura.³⁷

In the case of Georgi Markov,³¹ the lethal injected dose was estimated to be as much as 500 μg . This resulted in almost immediate local pain, followed by general weakness within about 5 hours. Fifteen to 24 hours later, he exhibited elevated temperature, nausea, and vomiting. Thirty-six hours after the incident, he was admitted to the hospital feeling ill and exhibiting fever and tachycardia. Blood pressure was 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 on his thigh. Just over 2 days after the attack, he suddenly became hypotensive and tachycardic. His pulse rate increased to 160 beats per minute, and white blood count rose to 26,300/ mm^3 .

Early on the third day, he became anuric and began vomiting blood. An electrocardiogram demonstrated complete atrioventricular conduction block. Markov died shortly thereafter. At the time of death, his white blood count was 33,200/ mm^3 . A mild pulmonary edema was thought to have been secondary to cardiac failure.

Inhalation

Although data on aerosol exposure to ricin in humans are not available, lesions induced by oral and parenteral exposure are consistent with those from animal studies, suggesting that the same would hold true for aerosol exposures. In humans, an allergic syndrome has been reported in workers exposed to castor bean dust in or around castor oil-processing plants.³⁸ The clinical picture is characterized by the sudden onset of congestion of the nose and throat, itchiness of the eyes, urticaria, and tightness of the chest. In more severe cases, wheezing can last for several hours, and may lead to bronchial asthma. Affected individuals respond to symptomatic therapy and removal from the exposure source. These patients may have had castor bean-positive skin prick tests, possess specific IgE against castor beans by the radioallergosorbent test technique, and may also have responded to a nasal challenge test with castor bean pollen.³⁹ It is likely, however, that these responses occurred as a result of exposure to bean constituents other than ricin.

Studies in mice demonstrate that aerosolized ricin is deposited in the trachea and lungs. This is followed by a decrease in detectable ricin in the lung and an increase in the trachea, likely due to pulmonary clearance via the mucociliary escalator. Pulmonary deposition is highly dependent upon aerosol particle size, which profoundly affects lethality in this animal model.⁴⁰ Immunohistochemistry studies in rats exposed to ricin by aerosol indicate that aerosolized ricin binds to ciliated bronchial cells, alveolar macrophages, and alveolar lining cells⁴¹ (Figure 15-2). Inhalational exposure of rats results in a diffuse necrotizing pneumonia of the airways, with interstitial and alveolar inflammation as well as edema.⁴² No notable changes in lung injury parameters occur before 8 hours postchallenge. By 12 hours, inflammatory cell counts and total protein (both from fluid obtained via bronchoalveolar lavage) increase, suggesting both enhanced permeability of the air-blood barrier and cytotoxicity. These findings are associated with a blood-cell analysis indicating inflammation. By 18 hours postchallenge, alveolar flooding is present, and extravascular lung water is increased. Both continue to increase for up to 30 hours. At 30 hours postchallenge, arterial hypoxemia and acidosis

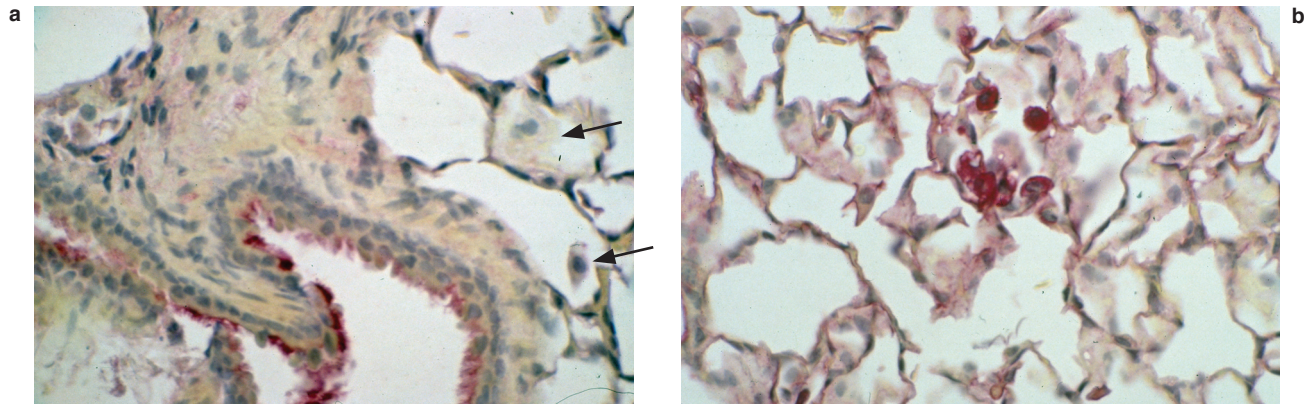


Fig. 15-2. Lung from a rat exposed to ricin by aerosol. Immunocytochemical stain for ricin demonstrates strong reactivity for (a) airway epithelial cells and alveolar macrophages (arrows) and (b) alveolar lining cells (immunocytochemical stain, original magnification $\times 50$). Photographs: Courtesy of Lieutenant Colonel CL Wilhelmsen, DVM, PhD, Veterinary Corps, US Army, Division of Pathology, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

are present, and histopathological evidence of alveolar flooding becomes significant.

Inhalation toxicity in nonhuman primates is characterized by a dose-dependent preclinical period of 8 to 24 hours, followed by anorexia and progressive

decrease in physical activity. Death occurs 36 to 48 hours postchallenge and is dose-dependent. Relevant gross and histopathological changes are confined to the thoracic cavity (Figure 15-3). All monkeys in this study developed acute marked-to-severe fibrinopurulent

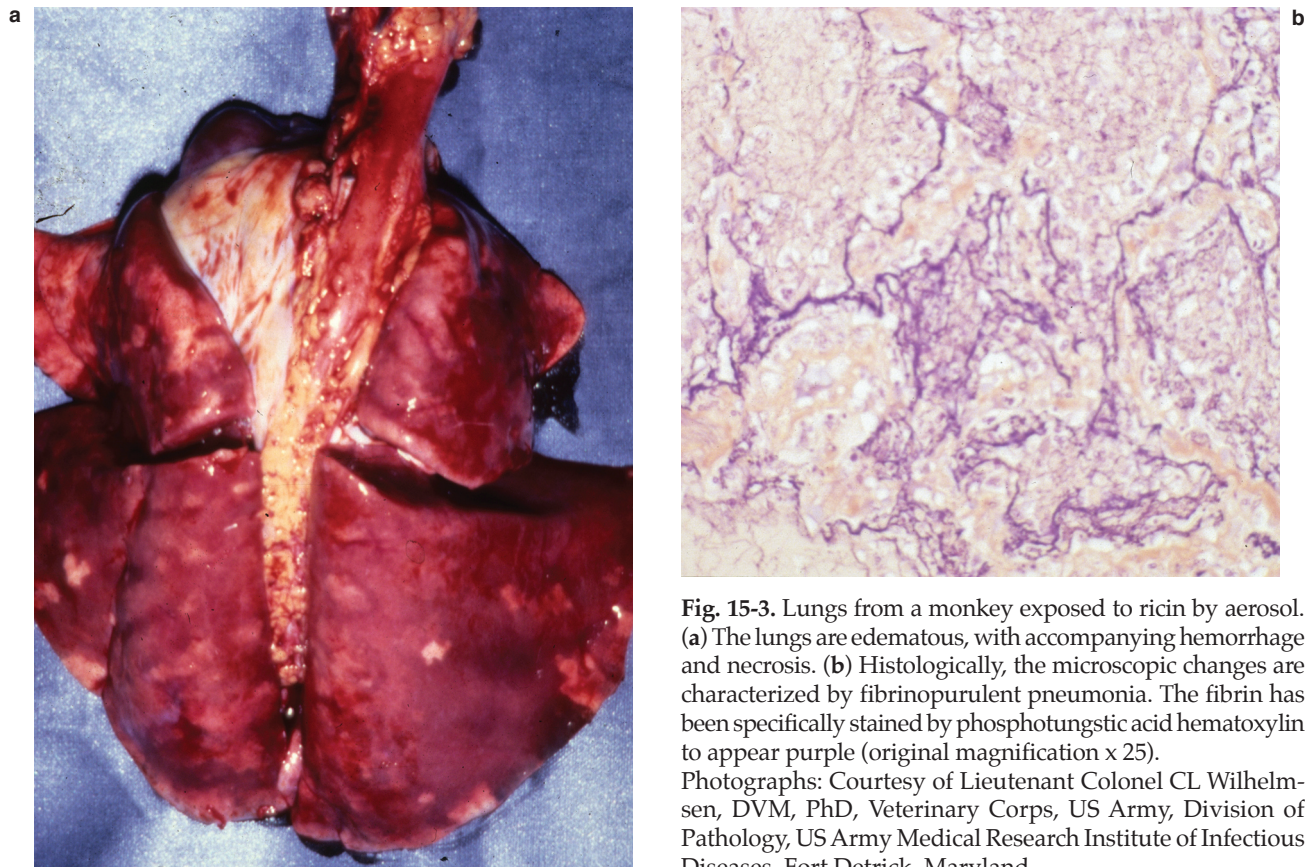


Fig. 15-3. Lungs from a monkey exposed to ricin by aerosol. (a) The lungs are edematous, with accompanying hemorrhage and necrosis. (b) Histologically, the microscopic changes are characterized by fibrinopurulent pneumonia. The fibrin has been specifically stained by phosphotungstic acid hematoxylin to appear purple (original magnification $\times 25$). Photographs: Courtesy of Lieutenant Colonel CL Wilhelmsen, DVM, PhD, Veterinary Corps, US Army, Division of Pathology, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

pneumonia, with variable degrees of diffuse necrosis and acute inflammation of airways. There were also diffuse, severe alveolar flooding and peribronchovascular edema (Figure 15-4), acute tracheitis, and marked-to-severe purulent mediastinal lymphadenitis. Two monkeys had acute adrenalitis.⁴³

Cause of Death

The exact cause of death is unknown and probably varies with route of intoxication. Ingesting the toxin results in ulceration and hemorrhage of the stomach and small intestine mucosa, necrosis of the mesenteric lymphatics, liver necrosis, nephritis, and splenitis. Resultant loss of fluid and electrolytes may lead to hypotension, tachycardia, dehydration, cyanosis, and vascular collapse. Injection of the toxin may lead to severe local lymphoid necrosis, gastrointestinal hemorrhage, liver necrosis, diffuse nephritis, and diffuse splenitis. High doses administered intravenously in laboratory animals are associated with disseminated intravascular coagulation, and it has been suggested that hepatocellular and renal lesions result from vascular disturbances induced by the toxin rather than a direct effect of the toxin itself.⁴⁴ Early studies^{45,46} clearly established that intravenous administration of ricin to rats results in diffuse damage to Kupffer cells within 4 hours, followed by endothelial cell damage, formation of thrombi in the liver vasculature,

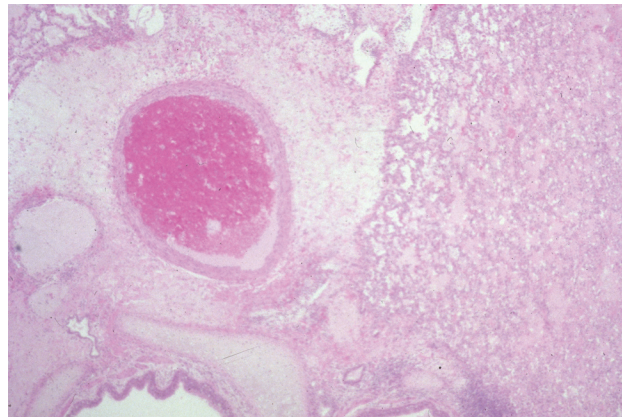


Fig. 15-4. Widespread perivascular and peribronchiolar edema in a monkey, a characteristic finding in aerosol ricin intoxication (hematoxylin-eosin stain, original magnification $\times 10$).

Photograph: Courtesy of CL Wilhelmsen, DVM, PhD, Lieutenant Colonel, Veterinary Corps, US Army, Division of Pathology, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

and finally, hepatocellular necrosis. In mice, rats, and primates, high doses by inhalation apparently produce lethal pulmonary damage, probably due to hypoxemia resulting from massive pulmonary edema and alveolar flooding.

DIAGNOSIS

As with other potential intoxications on the unconventional battlefield, epidemiological findings will likely play a central role in diagnosis. The observation of multiple cases involving severe pulmonary distress in a population of previously healthy young soldiers, linked with a history of being at the same place and time during climatic conditions suitable for a biological warfare attack, would suggest an aerosol exposure. Additionally, ingestion should be suspected in the case of several soldiers with gastrointestinal hemorrhage and hypotension who have eaten from the same food source. In patients who may be targets of an assassination attempt, ricin injection should be considered if there are signs of rapid onset of symptoms similar to VLS.

The differential diagnosis of aerosol exposure to ricin should include staphylococcal enterotoxin B and exposure to pyrolysis by-products of organofluorine polymers, such as Teflon and Kevlar (both manufactured by DuPont, Wilmington, Del), or other organohalides, oxides of nitrogen, and phosgene. Insecticides, although not expected in a battlefield scenario, can

be spread aurally over large geographical areas and should be included in the differential diagnosis. The differential diagnosis of ingested ricin includes enteric pathogens, enterotoxins, and other toxins, including caustic agents, mushroom species, hydrocarbons, and pharmaceuticals such as salicylates and colchicine.

After inhalational intoxication in laboratory animals, findings are generally nonspecific. Confirmation of inhalational intoxication in humans would most likely be through immunological analysis of a swab sample from the skin or nasal mucosa. Ricin can be identified from such samples by immunoassay for at least 24 hours postexposure.⁴⁷ Because ricin is extremely immunogenic, individuals surviving a ricin attack would likely have circulating antibody within 2 weeks of exposure. Therefore, serum samples should be obtained from survivors. Immunoassay of blood or other body fluids may be useful for confirming ricin intoxication. This test can accurately measure ricin to less than 1 ng/mL in clinical matrices.⁴⁸ However, because ricin is bound very quickly regardless of exposure route and metabolized before excretion,⁴⁹ identification in body

fluids or tissues is difficult. Although analytical methods for detecting the toxin are available from reference labs, including the US Army Medical Research Institute of Infectious Diseases and the Centers for Disease Control

and Prevention there are no clinically validated methods to detect ricin in biological fluids.⁵⁰ Postmortem identification of toxin in tissues can be accomplished through immunohistochemistry.

MEDICAL MANAGEMENT

The potential scenarios in which ricin intoxication might be seen by military medical personnel are: (a) small-scale battlefield or terrorist delivery of an aerosol; (b) parenteral administration of the toxin to an individual by an assassin's tool; or (c) contamination of food sources. Because ricin acts rapidly and irreversibly (directly on lung parenchyma after inhalation or distributed quickly to vital organs after parenteral exposure), postexposure therapy is difficult. Therefore, vaccinating personnel at risk for ricin exposure is an important consideration.

Vaccination and Passive Protection

Inhalational exposure is best countered with active vaccination. However, there is currently no licensed vaccine available. Development of a ricin vaccine has previously focused on either a deglycosylated ricin A chain or formalin-inactivated toxoid.⁵¹ Both preparations confer protection against aerosolized ricin. However, ricin is not completely inactivated by formalin and may retain some of its enzymatic activity (albeit approximately 1,000-fold lower than native ricin). Deglycosylated ricin A chain may lead to local or systemic VLS.

More recent research has evaluated recombinant ricin A chains to eliminate toxicity and improve the stability of the vaccine. An optimized vaccine candidate, RTA 1-33/44-198, was developed by the US Army by structurally modifying the ribosome-inactivating protein fold to create a nonfunctional scaffold for presentation of a specific protective epitope. This vaccine candidate protected 100% of vaccinated animals against supralethal aerosol challenges.⁵² Other mutants of recombinant ricin A chains devoid of enzymatic activity have also been developed. However, these mutants may still induce VLS in vaccinated individuals. A vaccine candidate based on a mutation of both the enzymatic site and the VLS-inducing site has been developed by a research group in Texas.⁵³ This candidate, RiVax (DOR BioPharma, Inc, Miami, Fla) is at least 10,000-fold less active than wild-type ricin A chain regarding inhibition of protein synthesis, and does not induce VLS. RiVax also protected mice against intraperitoneal challenge of up to 10 median lethal doses. The producer is now beginning phase I clinical trials to assess the safety and immunogenicity

of RiVax in humans.

Passive protection with aerosolized anti-ricin immunoglobulin (IgG) has also been evaluated as prophylaxis before aerosol challenge. Administration of nebulized anti-ricin IgG effectively protected against lung lesions and lethality in mice when challenged with an aerosol exposure to ricin approximately 1 hour later. Extrapolation of these data to clearance rates of IgG from the airways of rabbits suggests that anti-ricin-specific antibodies may provide protection for up to 2 to 3 days or longer.⁵⁴ These findings suggest that inhaling protective antibody from a portable nebulizer just before an attack might provide some protection in nonimmune individuals. However, the window of opportunity for treatment by intravenous administration or inhalation of specific antibody after exposure is probably minimal at best.

Supportive and Specific Therapy

The route of exposure for any agent is an important consideration in determining prophylaxis and therapy. For oral intoxication with ricin, supportive therapy includes intravenous fluid and electrolyte replacement and monitoring of liver and renal functions. Standard intoxication principles should be followed. Gastric lavage, if not contraindicated, may help to remove the toxin. Activated charcoal should be considered. The degree of adsorption of ricin by activated charcoal is unknown, and it may be minimally effective given the molecular size of the toxin. Percutaneous exposures require judicious use of intravenous fluids and monitoring for symptoms associated with VLS, including hypotension, edema, and pulmonary edema. Supportive care should entail correction of coagulopathies, respiratory support, and monitoring for liver and renal failure. For inhalational intoxication, supportive therapy to counteract acute pulmonary edema and respiratory distress is indicated. Symptomatic care is the only intervention presently available to clinicians for treating incapacitating or potentially lethal doses of inhaled ricin. Positive-pressure ventilator therapy, fluid and electrolyte replacement, antiinflammatory agents, and analgesics would likely be of benefit in treating the aerosol-exposed patient. A variety of chemotherapeutic agents—including cellular membrane effectors,

calcium channel–blocking agents, sodium-calcium exchangers, reducing agents, antioxidants, effectors of endocytosis, nucleoside derivatives, antibacterials, ricin analogs, effectors of cellular metabolism, and binding inhibitors—have been systematically screened

in in-vitro and in-vivo models for efficacy against ricin toxicity. However, no compounds were identified that could protect against lethality *in vivo*, and only two compounds, dexamethasone and difluoromethyornithine, extended survival times in mice.^{55,56}

SUMMARY

Ricin is a type II RIP toxin derived from the castor bean plant *R communis*. The plant is globally distributed in tropical and subtropical climates, and the beans are a major agricultural commodity in several countries. This agricultural production, coupled with the ease of toxin extraction, results in the potential availability of large quantities of ricin. Ricin was developed as an aerosol biological weapon by the United States and its allies during World War II, although it was never used in battle. In recent years, the threat of ricin on the battlefield has diminished, while its threat as a potential weapon of bioterror has increased. Although toxic by several routes, the greatest physiological threat is by inhalation. Contamination of the food supply is a lesser

threat due to much lower potency by this route.

Signs and symptoms of ricin exposure are route- and dose-dependent. Inhalation probably causes death by hypoxia secondary to massive pulmonary edema and alveolar flooding. Diagnosis is based upon both epidemiological and clinical parameters; laboratory confirmation of clinical samples is possible by immunoassay but complicated by pharmacokinetic factors. Treatment is purely supportive. Prophylaxis will be best accomplished by vaccination, although no vaccine is currently available. However, excellent vaccine candidates based upon genetically-engineered recombinant A chains are currently in advanced development or clinical trials.

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Chapter 16

BOTULINUM TOXIN

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INTRODUCTION

The neurotoxins produced by *Clostridia* species are among the most potent toxins known. Because of their extreme toxicity, botulinum (*C botulinum*) neurotoxins were 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 16-1). Botulism is a neuroparalytic disease, most commonly caused by foodborne ingestion of neurotoxin types A, B, and E, and is often fatal if untreated.

HISTORY

In the early 1930s, during its occupation of Manchuria, Japan formed a biological warfare command called Unit 731. General Shiro Ishii, the military medical commander of Unit 731, admitted to feeding lethal cultures of *C botulinum* to prisoners.¹ US researchers began working on weaponization of botulinum toxin in the 1940s, and Allied intelligence indicated that Germany was attempting to develop botulinum toxin as a weapon to be used against invasion forces.² 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 to determine its pathogenesis. The potential of botulinum neurotoxin as an offensive biological weapon was also investigated³⁻⁵ (the US code name for botulinum neurotoxin was "agent X").

Following President Richard M Nixon's executive orders in 1969–1970, all biological agent stockpiles in the US offensive biological program, including botulinum neurotoxin, were destroyed. 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.

Although the Soviet Union signed and ratified the convention,⁶ its biowarfare program, including botulinum neurotoxin research, weapons development, and production, continued and was even expanded in the post-Soviet era.^{7,8} The Soviet Union reportedly tested botulinum-filled weapons at the Soviet site Aralsk-7 on Vozrozhdeniye (Renaissance) Island in the Aral Sea^{8,9} and also attempted to use genetic engineering technology to transfer complete toxin genes into other bacteria.¹⁰ In April 1992, President Boris Yeltsin publicly declared that his country had covertly continued a massive offensive biological warfare buildup, which included developing botulinum toxin as a weapon. That same year, Colonel Kanatjan Alibekov (Kenneth Alibek), 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 described in detail the Soviet biological weapons program.¹⁰

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 4,900 gallons of concentrated botulinum neurotoxin for use in specially designed missiles, bombs, and tank sprayers in 1989 and 1990.^{7,11} Of this preparation, 2,600 gallons were used to fill 13 SCUD missiles with a 600-km range and 100 400-lb (R-400) bombs (each bomb could hold 22 gallons 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.¹²

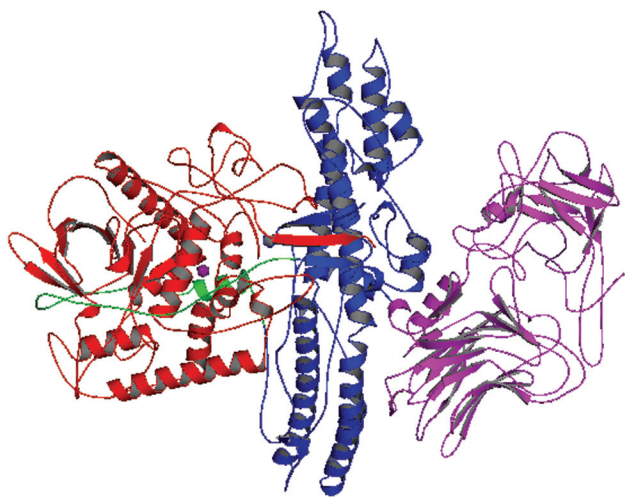


Fig. 16-1. Botulinum neurotoxin A is composed of an ~50 kDa light chain (LC-red) and an ~100 kDa heavy chain linked by a single disulfide bond. The LC functions as a zinc-dependent endopeptidase, whereas the heavy chain contains two functional ~50 kDa domains: a C-terminal ganglioside binding domain (Hc-purple), and an N-terminal translocation domain (Hn-blue). A belt portion of Hn (green) wraps around LC. The active site zinc is shown as a purple sphere. This figure is based on the structure determined by Lacy and colleagues. Data source: Lacy DB, Tepp W, Cohen AC, DasGupta BR, Stevens RC. Crystal structure of botulinum neurotoxin type A and implications for toxicity. *Nat Struct Biol.* 1998;5:898–902.

Courtesy of S Ashraf Ahmed, MD, Integrated Toxicology Division, US Army Medical Research Institute of Infectious Diseases, Fort Detrick, Maryland.

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. Before the sarin attack, three briefcases containing portable disseminating devices generating water vapor were found in the subway station. 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.¹³ Although no cult members were specialists in biological weapons development, microbiologists, medical doctors, and other scientists were among the followers. It is not fully understood why the biological assaults failed, but information from Asahara's trial indicated that the cult's scientists had difficulty overcoming technical barriers in isolating and cultivating *C botulinum*.¹³

A successful bioterrorist attack on large numbers of people with botulinum neurotoxin would likely 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 had used a colorless, odorless, and tasteless solution of botulinum toxin instead of *Salmonella typhimurium* on salad bars in its 1984 attack in The Dalles, Oregon,¹⁴⁻¹⁶ 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.¹⁷

In 2005 Wein and Liu¹⁸ described in detail how a bioterrorism attack using botulinum neurotoxin could be perpetrated upon the nation's 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.¹⁸ Naturally occurring salmonellosis outbreaks from milk and milk products affecting over 200,000 persons have already occurred, leading to a realistic assessment of such vulnerability in the national milk distribution system.^{19,20} 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,²¹ based upon microbiological contamination at a recreational facility²²; however, Wein and Liu's modeling goes much further than toxin generation, pinpointing critical entry points of neurotoxin into the milk supply, estimating the amount of toxin required, and pointing out weaknesses in current detection technology.¹⁸ The paper has generated debate.²³ Stewart Simonson, former assistant secretary for public health emergency preparedness at the US Department of Health and Human Services, has regretted the publication decision.²⁴

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 the intestinal tract of domestic grazing animals.²⁵⁻²⁹ 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 the canning or preservation of foods. The spores are hardy, and special efforts in sterilization are required to ensure that the spores are inactivated. Modern commercial procedures have virtually eliminated food poisoning by botulinum toxin; most cases today are associated with home-canned foods (particularly vegetables such as beans, peppers, carrots, and corn that are associated with a higher pH) or food items prepared by restaurants.^{30,31}

C botulinum produces seven antigenic types of neu-

rotoxins, denoted by the letters A through G. All seven neurotoxins are structurally similar (approximately 150 kd in mass) but immunologically distinct.³² 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.³³ The unique strain *C baratii* produces only serotype F,³⁴ and the *C butyricum* strain, serotype E.³⁵

Botulism is a neuroparalytic disease. Human botulism cases are caused primarily by neurotoxin types A, B, and E,³⁰ and occasionally by type F.³⁶ *C argentinense* produces type G, which has been associated with sudden death, but not neuroparalytic illness, in a few patients in Switzerland.³⁷ Types C and D cause disease in animals. All seven toxins are known to cause inhalational botulism in primates,³⁸ and therefore could potentially cause disease in humans. Clostridial C2 cytotoxin is an enterotoxin, but not a neurotoxin. 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.^{39,40}

Botulinum Neurotoxin Production

Spore germination and subsequent growth of toxin-producing bacteria occur in improperly preserved foods,^{41–48} decaying animal carcasses and vegetable matter,^{49–53} and microbiology laboratories.^{54–58} A ter-

rorist 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. The 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 (discussed later in this chapter).

PATHOGENESIS

The seven neurotoxins have different specific toxicities^{59–61} and durations of persistence in nerve cells.^{62,63} All botulinum toxin serotypes inhibit acetylcholine release, but they act through different intracellular protein targets, exhibit different durations of effect, and have different potencies.⁶⁴ 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), and from infected wounds (wound botulism). Upon absorption, the circulatory system transports the toxin to peripheral cholinergic synapses, primarily targeting

neuromuscular junctions.⁶⁵ 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,^{66,67} and noncholinergic cells may also be affected.⁶⁸ The estimated human dose (assuming a weight of 70 kg) of type A toxin lethal to 50% of an exposed population (the LD₅₀) 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.^{69–72}

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.^{30,73} In foodborne botulism, symptoms appear several hours to within a few days (range 2 hours to 8 days) after contaminated food is consumed.³⁰ 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.⁷³ However, the median time to onset of symptoms for serotype E was much 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.⁷³

As a neuroparalytic illness, botulism presents as an acute, symmetrical, descending, 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 lastly muscles of the lower extremities. Weakness of the extremities generally occurs in a proximal-to-distal pattern, and is generally symmetric.³¹ However, asymmetric extremity weakness may occasionally be observed, occurring in 9 of 55 botulism cases in one review.⁷⁴ 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.⁷³ Other commonly reported symptoms include fatigue, sore throat, dry mouth, constipation, and dizziness.⁷⁴ 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.⁷⁴ 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.^{73–75} 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.⁷⁴ Symptoms of cranial nerve dysfunction and mild autonomic dysfunction may persist for more than a year.⁷⁶⁻⁷⁸

The following symptom triad should suggest the diagnosis of botulism: (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.⁷⁴ However, individuals may not exhibit all five symptoms; a recent review from the Republic of Georgia reported that only 2% of patients (13/481) presented with all five criteria.⁴⁸

Although foodborne botulism is the most likely route of exposure for botulism from natural causes or a bioterrorist event, botulism acquired on the battlefield is most likely to occur from inhalation of botulinum toxin, 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.^{73,79} Clinical symptoms resulting from inhalational intoxication are similar to botulism acquired from ingestion of the toxin.

The only reported inhalation-acquired botulism in humans occurred in 1962 in a German research laboratory.⁸⁰ 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 antbotulinum 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.⁸⁰

DIAGNOSIS

The differential diagnosis of botulism includes other diseases with symptoms of paralysis:

- Guillain-Barré syndrome (usually ascending paralysis, paresthesias common, elevated cerebrospinal fluid (CSF) protein [may be normal early in illness], electromyogram findings). Note: 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.⁷⁴
- Myasthenia gravis (dramatic improvement with edrophonium chloride, autoantibodies present, electromyogram findings). Note: Botulism cases may have a positive response to edrophonium chloride (26%), but the response is generally not dramatic.⁷⁴
- 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).

The clinical presentation of an afebrile patient with an acute, symmetric, descending, flaccid paralysis (without sensory deficits) with a normal sensorium suggests the diagnosis of botulism. 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.⁸¹⁻⁸³ An electromyogram with repetitive nerve stimulation at 20 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 Eaton-Lambert syndrome.³¹ 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.⁷⁴ 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 (17%) who had spinal fluid analysis performed.⁷⁴ 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.

Foodborne Botulism

In foodborne botulism, a confirmatory diagnosis can often be made by demonstrating the presence of toxin in patient specimens, such as the serum, stool, gastric aspirate, or vomitus, using mouse bioassays. Mouse bioassays are performed by injecting mice intraperitoneally with the specimen sample suspected to contain toxin (with and without antitoxin). If toxin is present in the specimen, mice injected with the specimen alone (without antitoxin) will usually die from botulism within 6 to 96 hours, but mice injected with the specimen treated with 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.

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.

Toxin Assays in Foodborne Botulism

Toxin assays of specimens from cases of foodborne botulism from 1975 to 1988 showed the presence of toxin in specimens from various sites as follows:

sera, 37% (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.⁷³ 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.⁷³

Cultures in Foodborne Botulism

Stool and gastric aspirate cultures for *C botulinum* resulted in a higher yield of diagnosis than toxin assays.⁷³ 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. This 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. 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.⁸⁴

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.^{85,86} In inhalational exposures to botulinum toxin, the toxin may be detected in the nares for up to 24 hours after exposure, using either an enzyme-linked immunosorbent assay or polymerase chain reaction test of a nasal mucosal swab.^{86,87} However, these tests have limited validation in botulism diagnosis, and they may not be as sensitive as mouse neutralization assay in the detection of toxin. 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 inhalation of toxin preparations that do not contain spores of the organism.

TREATMENT

The current recommended treatment for botulism, although limited, includes antitoxin therapy and supportive care as needed, including mechanical ventilation. 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. Surgical debridement and antibiotic therapy are recommended for the treatment of wound botulism. 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.⁸⁸

Antitoxin

Mortality from botulism before 1950 was approximately 60%.³¹ In the early 1970s, therapy with equine antitoxins was introduced, and the botulism case fatality rate dropped to about 23%. The evidence for efficacy of botulinum antitoxin in humans is based on retrospective analyses of small numbers of patients and on animal studies. In one study, type A botulism was associated with a mortality rate in humans of 10% (3/30) with antitoxin therapy, versus 46% (6/13) without antitoxin therapy.⁷⁵ The fatality rate of botulism from toxin serotype E in humans was associated with a mortality rate of 3.5% with antitoxin therapy, versus 28.9% without antitoxins.⁸⁹ Although the evidence is limited, it is believed that early treatment, especially within 24 hours, is most effective in preventing progression of paralysis. 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 up to 12 hours after antitoxin administration before an effect is observed. With adequate ventilatory assistance, tracheostomy, and improved intensive care support, fatality rates from botulism are now less than 5% to 10%.

Individuals suspected to have been exposed to botulinum toxin should be carefully monitored. If a person begins to develop symptoms of botulism, botulinum antitoxin should be administered. Because most antitoxin preparations are equine products, there is a risk of hypersensitivity reactions. Skin testing must be performed before administering equine antitoxins, as described in the package insert. The bivalent botulinum equine antitoxin (serotypes A and B) is the only FDA-approved antitoxin preparation

currently available for adults, and may be obtained from the CDC (contact the local health department or, if it is unavailable, the CDC at 770-488-7100). The CDC also has an investigational equine antitoxin product for toxin serotype E. The trivalent equine botulinum antitoxin product (A, B, E) is no longer available at the CDC because of declining antitoxin titers to toxin serotype E in the product. An investigational human botulinum immune globulin against toxin serotype E is also available at the California Department of Health Services (510-231-7600).

In October 2003 the FDA approved human botulinum immune globulin (BabyBIG), a significant advancement in the treatment of infantile botulism. BabyBIG is a human botulinum immune globulin derived from pooled plasma of adults immunized with pentavalent botulinum toxoid, with subsequent development of high titers of neutralizing antibodies against toxins serotypes A and B. Because it is derived from humans, BabyBIG does not have the high risk of anaphylaxis observed with the equine products, nor the risk of lifelong hypersensitivity to equine antigens. Infantile botulism occurs primarily in newborns less than one year of age, caused by toxin production from intestinal colonization and growth of *C botulinum*, with approximately 100 cases diagnosed per year in the United States.⁹⁰ Use of BabyBIG is anticipated to save about \$70,000 per case in hospital costs.^{91,92} BabyBIG can be obtained from the California Department of Health Services.

Additionally, two equine antitoxin preparations against all seven toxin serotypes, developed by the US Army Medical Research Institute of Infectious Diseases, are available as investigational drugs for treating botulism: (1) botulinum antitoxin, heptavalent, equine, types A, B, C, D, E, F, and G (HE-BAT) and (2) botulinum antitoxin, F(ab')₂ heptavalent, equine toxin neutralizing activity types A, B, C, D, E, F, and G (Hfab-BAT). These products are "despeciated" equine antitoxin preparations, made by cleaving the Fc fragments from the horse immunoglobulin G molecules to reduce the side effects such as serum sickness and hypersensitivity reactions, leaving only the F(ab')₂ fragments. Although the species-specific antigens have been removed, there is still a reduced risk for hypersensitivity reactions because 4% of horse antigen molecule remains in the preparation. The HE-BAT heptavalent product, when administered to an individual as a single vial of 10 mL, was formulated

to provide more than 4,000 IU of serotypes A, B, C, E, and F, and more than 500 IU of serotypes D and G antitoxin. One IU (international unit) of antitoxin, by definition, is the amount of antitoxin that will neutralize 10,000 LD₅₀ of toxin serotypes A, B, C, D, F, and G, respectively, and 1,000 LD₅₀ of toxin serotype E. These investigational products would be considered for treatment of botulism in the event of biowarfare or bioterrorism, which may involve the use of any of the seven toxin serotypes.

Animal studies show that the heptavalent antitoxin products are protective in both mice and nonhuman primates. The products were shown to neutralize each of the botulinum toxin serotypes *in vitro*; mice injected with a mixture of heptavalent antitoxin and a specific toxin serotype did not develop symptoms of botulism. The Hfab-BAT product, given to asymptomatic mice within a few hours after aerosol challenge with approximately 10 LD₅₀ 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.⁷² The product was also protective against aerosol challenge to toxin serotype A at a dose of approximately 2,000 mouse intraperitoneal LD₅₀/kg, when given to nonhuman primates immediately prior to exposure (protection in 5/5 animals), and when given 48 hours after inhalational exposure (protective in 3/5 monkeys).

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 within 2.8 to 11 hours postexposure, much earlier than observed in humans and nonhuman primates, in which death generally does not occur until 2 to 3 days postexposure. In one review of foodborne botulism in humans, 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.⁴⁸

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 must be given early to have

a beneficial effect, the initial diagnosis of botulism is clinical, with confirmation by laboratory findings afterwards. Neurological signs and symptoms resulting from a toxin-induced blockade of neurotransmission at voluntary motor and cholinergic junctions dominate the clinical manifestation of botulism.^{73,93,94} A diagnosis of botulism is suggested in individuals presenting with an acute onset of cranial nerve weakness (ie, diplopia, 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 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, Calif] contains only 100 units of toxin) was used by an unlicensed physician for cosmetic injections into three patients and himself.^{95,96} The four individuals were subsequently admitted to medical facilities with symptoms of botulism and faced a long-term recovery.⁹⁷

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 makes preexposure or postexposure prophylaxis with these agents impractical for large numbers of persons.^{85,98} Administration of equine antitoxin is not recommended for preexposure prophylaxis, due to 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.

There are currently no FDA-approved vaccines for the prevention of botulism. However, an investigational product, the pentavalent botulinum toxoid (PBT) against botulinum toxin serotypes A through E, has been used since 1959 for persons at risk for botulism (ie, laboratory workers)⁹⁹⁻¹⁰¹ and is available as an investigational product on protocol through the CDC. 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, Mich). 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 adjunct. The five serotypes were then blended together to produce the end product, in a formulation based on concentrations that induce protective immunity in guinea pigs against a lethal challenge of 10^5 mouse intraperitoneal LD₅₀ of each of the respective toxin serotypes. The Michigan Department of Community Health is responsible for formulation of current lots of PBT. The final product is bottled in 5-mL multidose vials, each containing 0.22% formaldehyde as a stabilizer and thimerosal in a 1:10,000 ratio as a preservative. Each 0.5-mL dose of vaccine contains 7 mg of aluminum phosphate and approximately 5 μ g of inactivated toxin.

PBT has been found to be protective in animal models against challenge with botulinum toxin serotypes A through E,¹⁰² including protection in nonhuman primates against aerosol challenge to toxin serotype A.¹⁰³ At-risk laboratory workers in the US offensive biological warfare program at Fort Detrick were immunized with a bivalent botulinum toxoid (serotypes A and B) beginning in 1945, and then with PBT beginning in 1959.¹⁰¹ 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.

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.¹⁰⁴ Since 1993, and until recently, booster doses subsequent to the 12-month booster were administered only for declining titers (no detectable titer on a 1:16 dilution of serum, corresponding to approximately 0.25 IU/mL for toxin serotype A).¹⁰⁵ The PBT dosing schedule was changed in 2004 due to (a) a recent decline in PBT immunogenicity and potency noted on the yearly potency testing, and (b) data from a 1998–2000 PBT study found a decrease in antitoxin

titers by week 24 (6 months) in approximately two thirds of vaccinees.^{106,107} The protocol for PBT (for current lots produced in the 1970s, which are now 30 years of age) now requires a primary series of four injections (0.5 mL at 0, 2, 12, and 24 weeks), followed by a booster dose at 12 months (because the 1998–2000 PBT study showed that antitoxin titers after the 24-week dose declined again by month 12), and booster doses annually thereafter.¹⁰⁶⁻¹⁰⁸

Results of the potency testing are consistent with results of antitoxin titers obtained at the US Army Medical Research Institute of Infectious Diseases from 1999 to 2001. The PBT showed continued induction of antitoxin titers to toxin serotype A (≥ 0.02 IU/mL) in nearly all vaccinees (30/32, or 94%) at 28 to 56 days after completion of the initial three doses of PBT.¹⁰⁵ Titers to toxin serotype A even lower than 0.02 IU/mL have been shown to provide protection in nonhuman primates.¹⁰³ However, only one of seven persons had detectable antitoxin titers to toxin serotype E between day 28 and 56 after completion of the initial three PBT doses.¹⁰⁵ Additionally, although PBT booster doses resulted in higher titers to toxin serotype A (≥ 0.32 IU/mL) in 96% (47/49) of the vaccinees at 28 days after the booster, as well as persistent titers—95% (35/37) of vaccinees had detectable titers (> 0.02 IU/mL) and 76% (28/37) had higher titers (≥ 0.32 IU/mL) at 6 to 12 months—detectable titers to toxin serotype E after a booster dose were observed in only 42% (10/24) of vaccinees at 6 to 12 months.¹⁰⁵

PBT has been 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.⁹⁹ The main adverse event has been local reactions. Adverse events passively reported to the CDC between 1970 to 2002 for over 20,000 vaccinations included moderate local reactions (edema or induration between 30 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%.¹⁰⁸

PBT is not useful or recommended for postexposure prophylaxis because measurable antitoxin titers do not usually occur until a month after the third dose of the vaccine (4 months after the first vaccine dose).^{104,105,109,110} PBT may be considered for preexposure prophylaxis in at-risk persons (ie, laboratory workers or military troops at high risk of a biowarfare exposure), but not in the general population, for whom the risk of botulinum intoxication is low. Additionally, the requirement

of multiple doses to maintain titers, the status of the vaccine as an investigational new drug, and the limited supply of the vaccine make the product difficult to use in large numbers of persons in an emergency setting. Today nearly all stocks of these products are held either by the US Army or the CDC for the pharmaceutical strategic national stockpile.

New Vaccine Research

Vaccine candidates include formalin-inactivated toxoids (tetraivalent [ABEF] and monovalent [F] products) made nearly the same way as formalin-inactivated PBT, with the goal of FDA approval.^{111,112} Production of formalin-inactivated toxoids requires handling biohazards, and there is a possibility of toxin reactivation in vivo.¹¹¹ However, the risk of reactivation may not be expected to be different from other toxoids, such as tetanus and diphtheria toxoids, that are FDA approved. Production also requires partially purified culture supernatants to be exhaustively treated with formaldehyde, which must be performed by a highly trained staff and within a dedicated high-containment laboratory space.¹¹³ Furthermore, the resulting PBT is relatively impure, containing only 10% neurotoxin (90% is irrelevant material). This impurity may contribute significantly to the occurrence of local reactions and to the need for multiple injections to both achieve and sustain protective titers.

The use of pure and concentrated antigen in recombinant vaccines could offer the advantages of increased immunogenicity and a decrease in reactogenicity (local

reactions at the injection site) over formalin-inactivated toxoids.¹¹⁴ Recombinant techniques use a fragment of the toxin that is immunogenic, but does not have the capability of blocking cholinergic neurotransmitters. Both *Escherichia coli* and yeast expression systems have been used in the production of recombinant fragments, mainly the carboxy-terminal fragment (H_c) of the heavy chain of the toxin. Vaccine candidates using recombinant fragments of botulinum toxins against serotypes A, B, C, E, and F were protective in mice.¹¹⁵⁻¹²³ A vaccine recombinant candidate for serotype A was protective in mice against intraperitoneal challenge and produced levels of immunity similar to that attained with PBT, but with an increase in safety and decrease in cost per dose.¹¹³ Recombinant vaccines given by inhalational route are also being investigated.^{124,125} Work at the US Army Medical Research Institute of Infectious Diseases led to the development of a new bivalent recombinant botulinum vaccine (toxin serotype A and B) that is currently undergoing phase I trials in humans.¹²⁶ The vaccine is administered as two doses (at 0 and 6 weeks). Preliminary review of the safety and immunogenicity data suggests that phase II trials with this vaccine may soon be proposed.

A candidate vaccine that involves the insertion of a synthetic carboxy-terminal fragment (H_c) gene of the heavy chain of toxin serotype A into the vector system of the Venezuelan equine encephalitis virus is also being evaluated.¹¹⁹ The vaccine induced a strong antibody response in the mouse model, and remained protective in mice against intraperitoneal challenge at 12 months.

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 a neuroparalytic disease, most commonly caused by foodborne ingestion of neurotoxin types A, B, and E, and is often fatal if untreated. Intoxication with neurotoxin type A may result in a more severe disease than from toxin serotypes B and E. Paralysis from botulism can be long-lasting, with concomitant demanding supportive care requirements. Clinicians should be able to recognize the classic symptoms of botulinum intoxication. Various laboratory assays for botulinum toxin are available for clinical

specimens, but patient treatment is initiated in the absence of laboratory confirmation, given an index of suspicion for botulism. Antitoxin therapy and supportive care are important for treating botulism patients. Bivalent (AB) botulinum equine antitoxin is the sole FDA-approved antitoxin for adults. Human botulinum immune globulin (BABY BIG) has recently been approved by the FDA and is available for the treatment of infantile botulism. PBT has been available for over 45 years as an investigational product for immunological protection against botulinum toxin; and two despeciated equine antitoxin preparations for toxin serotype A-G, an equine antitoxin for serotype E, and a human botulinum antitoxin against toxin serotype E are available as investigational drugs. Future vaccine research could lead to a new class of recombinant vaccines to protect against botulism.

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Chapter 17

ADDITIONAL TOXINS OF CLINICAL CONCERN

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INTRODUCTION

TRICHOTHECENE MYCOTOXINS

History

Description of the Toxin

Mechanism of Action

Clinical Signs and Symptoms of Intoxication

Diagnosis

Medical Management

MARINE ALGAL TOXINS

History

Paralytic Shellfish Poisoning

Neurotoxic Shellfish Poisoning

Amnesic Shellfish Poisoning

CLOSTRIDIAL TOXINS

History

Description of the Epsilon Toxin

Mechanism of Action

Clinical Signs and Symptoms

Medical Management

SUMMARY

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INTRODUCTION

Several toxins produced naturally by microorganisms and plants are potent, stable, and capable of causing a wide range of effects leading to incapacitation or death. These agents can be ingested, administered percutaneously, or potentially delivered as aerosols at the tactical level. Although these toxins may be lethal, the amount of toxin available from a single organism is typically small. Toxins listed on the Centers for Disease Control and Prevention's bioterrorism threat list are proteins of microbial or plant origin, and include *Clostridium botulinum* neurotoxin, *C perfringens* epsilon toxin, *Staphylococcus aureus* enterotoxin B, and ricin

from *Ricinus communis*. Additional, nonproteinaceous toxins that may pose a threat are the trichothecene mycotoxins (eg, T-2 toxin) and marine toxins (eg, saxitoxin [STX], brevetoxins, and domoic acid).

Although any of these toxins have the potential to cause significant effects in humans or animals, their potential as biological warfare/biological terrorism agents varies depending on several factors. These toxins are also clinically relevant because intoxications occur naturally in humans and animals. The toxins in this chapter have been selected for discussion because of their potential for intentional use.

TRICHOHECENE MYCOTOXINS

History

Mycotoxins are metabolites of fungi produced through secondary biochemical pathways. Various mycotoxins are implicated as the causative agents of adverse health effects in humans and animals that consumed fungus-infected agricultural products.^{1,2} Consequently, fungi that produce mycotoxins, as well as the mycotoxins themselves, are potential problems from a public health and economic perspective. The fungi are a vast group of eukaryotic organisms, but mycotoxin production is most commonly associated with the terrestrial filamentous fungi referred to as molds.³ Various species of toxigenic fungi are capable of producing different classes of mycotoxins, such as the aflatoxins, rubratoxins, ochratoxin, fumonisins, and trichothecenes.^{1,2}

Use in Biological Warfare

From 1974 to 1981 the Soviet Union and its client states may have used trichothecene toxins⁴ in Cold War sites such as Afghanistan, Laos, and Cambodia. These agents may have been delivered as an aerosol or droplet cloud by aircraft spray tanks, aircraft-launched rockets, bombs (exploding cylinders), canisters, a Soviet handheld weapon (DH-10), and booby traps. Alleged attacks in Laos (1975–1981) were directed against Hmong villagers and resistance forces who opposed the Lao People's Liberation Army as well as the North Vietnamese. In Afghanistan these weapons were allegedly delivered by Soviet or Afghan pilots against mujahideen guerrillas between 1979 and 1981. The attacks caused at least 6,310 deaths in Laos (226 attacks); 981 deaths in Cambodia (124 attacks); and 3,042 deaths in Afghanistan (47 attacks).⁵

The "Yellow Rain" Controversy

Some of the air attacks in Laos, described as "yellow rain," consisted of a shower of sticky yellow liquid that fell from the sky and sounded like rain. Other accounts described a yellow cloud of dust or powder, a mist, smoke, or an insect-spray-like material. More than 80% of the attacks were delivered by air-to-surface rockets and the remainder from aircraft-delivered sprays, tanks, or bombs.⁵ The use of other agents, such as phosgene, sarin, soman, mustards, CS gas, phosgene oxime, or BZ, has been suggested by intelligence information and symptoms described by the victims. These chemical agents may have been used in mixtures or alone, with or without the trichothecenes.⁵ Evidence for, and against, the use of trichothecenes in Southeast Asia has been fully discussed in previous texts.^{6,7,8}

Weaponization

Mycotoxins (especially T-2 toxin) have excellent potential for weaponization because of their antipersonnel properties, ease of large-scale production, and proven delivery by various aerial dispersal systems.^{5,7,11} In nanogram amounts, the trichothecene mycotoxins (in particular T-2 toxin) cause severe skin irritation (erythema, edema, and necrosis).^{8,11-15} It is estimated that T-2 toxin is about 400 times more potent in producing skin injury than mustard (50 ng for T-2 vs 20 μ g for mustard).⁹ Lower microgram quantities of trichothecene mycotoxins cause severe eye irritation, corneal damage, and impaired vision.^{4,5,9,16} Emesis and diarrhea have been observed at 0.1 to 0.2 lethal doses (LD) of trichothecene mycotoxins.⁹⁻¹⁹

By aerosol exposure, the lethality of T-2 toxin is 10 to 50 times greater than when it is injected parenterally,²⁰

depending upon the species and exposure procedure.²¹⁻²² With a larger dose in humans, aerosolized trichothecenes may produce death within minutes to hours.⁵⁻⁷ The inhaled toxicity of T-2 toxin is in the range of 200 to 5,800 mg/min/m³²⁰⁻²² and is similar to that observed for mustards or lewisite (range of 1,500–1,800 mg/min/m³).²³ Percutaneous lethality of T-2 toxin (median LD [LD₅₀] in the range of 2–12 mg/kg)^{9,14} is higher than that for lewisite (LD₅₀ of approximately 37 mg/kg) or mustards (LD₅₀ of approximately 4,500 mg/kg).²³

T-2 toxin can be produced by solid substrate fermentation at approximately 9 g/kg of substrate, with a yield of 2 to 3 g of crystalline product.²⁴ Several of the trichothecene mycotoxins have been produced in liquid culture at medium yields and large volumes of culture for extraction.²⁵ A trichothecene mycotoxin used in phase I and II cancer trials, 4,15-diacetoxyscirpenol (DAS), was produced large scale by a procedure considered proprietary by industry.¹⁰ Thus, using existing state-of-the-art fermentation processes developed for brewing and antibiotics, ton production of several trichothecene mycotoxins would be fairly simple.

The delivery methods allegedly used in Southeast Asia would result in a low-efficiency respiratory aerosol (1–5- μ m particles),²⁶ but a highly effective droplet aerosol could result in severe skin and eye irritation. A National Research Council/National Academy of Sciences expert committee estimated that the offensive use of trichothecene mycotoxins could produce concentrations of approximately 1 g/m³ in the exposure cloud and 1 g/m² on the ground.¹⁰ Much lower aerosol concentrations could be expected to cause significant incapacitating responses (ie, skin and eye irritation at nano/microgram quantities) that would adversely affect military operations.

Description of the Toxin

Natural Occurrence

Potentially hazardous concentrations of the trichothecene mycotoxins can occur naturally in moldy grains, cereals, and agricultural products.^{10,16} Toxicogenic species of *Fusarium* occur worldwide in habitats as diverse as deserts, tidal salt flats, and alpine mountain regions.¹⁰ A food-related mycotoxic disease has been recorded in Russia from time to time, probably since the 19th century.²⁷⁻²⁹ In the spring of 1932, this disease appeared in endemic form throughout several districts of western Siberia (with a mortality rate of up to 60%). From 1942 to 1947, more than 10% of the population in Orenburg, near Siberia, was fatally affected by overwintered millet, wheat, and barley.^{16,29,30} The syndrome

was officially named alimentary toxic aleukia (alternative names in the Russian literature include septic angina, alimentary mycotoxicosis, alimentary hemorrhagic aleukia, aplastic anemia, hemorrhagic aleukia, agranulocytosis, and Taumelgetreide [staggering grains]).^{27,29} Symptoms of this disease include vomiting, diarrhea, fever, skin inflammation, leukopenia, multiple hemorrhage, necrotic angina, sepsis, vertigo, visual disturbances, and exhaustion of bone marrow.^{27-29,31} Extensive investigations in Russia indicated that a toxin from *Fusarium* species was the causative agent of alimentary toxic aleukia.^{29,32,33} Subsequently, it was demonstrated that T-2 toxin, a potent trichothecene mycotoxin, was the likely agent of the disease.^{33,34}

Human cases of stachybotryotoxicosis (another toxic trichothecene mycotoxin) have been reported among farm workers in Russia, Yugoslavia, and Hungary.³⁵⁻³⁸ This disease is caused by a mold, *Stachybotrys atra*, on the hay fed to domestic animals. Symptoms of this toxicosis include conjunctivitis, cough, rhinitis, burning in the nose and nasal passages, cutaneous irritation at the point of contact, nasal bleeding, fever, and leukopenia in rare cases.^{35,36} A macrocyclic trichothecene (saratxin) is produced by *Stachybotrys* species, which may be partly responsible for this toxicosis.³⁷⁻⁴¹ The only apparent human cases of stachybotryotoxicosis in the United States cited in the literature occurred in people living in a water-damaged house heavily infested with *S atra*.⁴² Russian scientists have reported a case of “cotton lung disease” that occurred after inhalation of cotton dust contaminated with *Dendrodochium toxicum*, which is a fungus synonymous with *Myrothecium verrucaria* (a natural producer of the verrucarins class of macrocyclic trichothecenes).^{30,43}

The “red mold disease” of wheat and barley in Japan is prevalent in the region facing the Pacific Ocean.^{16,44} In humans, symptoms of this disease included vomiting, diarrhea, and drowsiness. Toxic trichothecenes, including nivalenol, deoxynivalenol, and monoacetylnivalenol (fusarenon-X), from *F nivale* were isolated from moldy grains.^{16,44} Similar symptoms were described in an outbreak of a foodborne disease in the suburbs of Tokyo, which was caused by the consumption of *Fusarium*-contaminated rice.¹⁰

In addition to human intoxication, ingestion of moldy grains contaminated with trichothecenes has also been associated with mycotoxicosis in domestic farm animals.^{30,31,44-51} Symptoms include refusal of feed, emesis, diarrhea, skin inflammation, hemorrhage, abortion, cyclic movement, stomatitis, shock, and convulsions. Overall, the symptoms evident in domestic farm animals that eat food contaminated with trichothecene mycotoxins are similar to those observed in humans.

Chemical and Physical Properties

The trichothecenes make up a family of closely related chemical compounds called sesquiterpenoids.¹⁶ All the naturally occurring toxins contain an olefinic bond at C-9,10, and an epoxy group at C-12,13; the latter characterizes them as 12,13-epoxy trichothecenes. The structures of approximately 150 derivatives of trichothecenes are described in the scientific literature.^{10,52,53} These mycotoxins are classified into four groups according to their chemical characteristics. The first two groups include the "simple" trichothecenes, and the other two include the "macrocylic" trichothecenes.^{16,30} Because of its relatively high toxicity and availability, T-2 toxin has been the most extensively studied trichothecene mycotoxin.

The trichothecene mycotoxins are nonvolatile, low-molecular-weight (250–550) compounds.⁵³ This group of mycotoxins is relatively insoluble in water; the solubility of T-2 toxin is 0.8 and 1.3 mg/mL at 25°C and 37°C, respectively.⁵⁴ In contrast, these toxins are highly soluble in acetone, ethylacetate, chloroform, dimethyl sulfoxide, ethanol, methanol, and propylene glycol.⁵³ Purified trichothecenes generally have a low vapor pressure, but they do vaporize when heated in organic solvents. Extracting trichothecene mycotoxins from fungal cultures with organic solvents results in a yellow-brown liquid, which, if allowed to evaporate, yields a greasy, yellow crystalline product believed to be the yellow contaminant of yellow rain. In contrast, highly purified trichothecenes form white crystalline products that have characteristic melting points.¹⁰

Trichothecene mycotoxins are stable compounds in air and light when maintained as crystalline powders or liquid solutions.^{10,54-57} When stored in sterile phosphate-buffered saline at pH 5 to 8 and 25°C, T-2 toxin was stable for a year, with an estimated half-life of 4 years.⁵⁴ In contrast, T-2 toxin degrades rapidly over several days in culture medium containing fetal bovine serum⁵⁸ or bacteria from soil or freshwater.⁵⁹ This suggests that enzymes present in serum or produced by bacteria can stimulate biotransformation of trichothecene mycotoxins. A 3% to 5% solution of sodium hypochlorite is an effective agent for inactivating trichothecene mycotoxins.^{56,57} The efficacy of this agent is increased by adding small amounts of alkali, but higher concentrations of alkali or acid alone do not destroy trichothecene activity. Thus, high pH environments are ineffective for inactivating trichothecene mycotoxins. The US Army decontaminating agents DS-2 and supertropical bleach inactivate T-2 toxin within 30 to 60 minutes. These mycotoxins *are not inactivated by autoclaving* (at 250°F for 15 minutes at 15 lb/in²); however, heating at 900°F for 10 minutes or 500°F for

30 minutes inactivates them.^{56,57} This emphasizes the marked stability of trichothecene mycotoxins under varying environmental conditions.

Mechanism of Action

The trichothecene mycotoxins are toxic to humans, other mammals, birds, fish, various invertebrates, plants, and many types of eukaryotic cells in general.^{1,2,8,10,30,60-62} The acute toxicity of the trichothecene mycotoxins varies somewhat with the particular toxin and animal species.^{8,10,43,60-63} Variations in species susceptibility to trichothecene mycotoxins are small compared to the divergence obtained by the diverse routes of toxin administration. Once the trichothecene mycotoxins enter the systemic circulation, regardless of the route of exposure, they affect rapidly proliferating tissues.^{8,10,16} Oral, parenteral, cutaneous, and respiratory exposures produce (a) gastric and intestinal lesions; (b) hematopoietic and immunosuppressive effects described as radiomimetic in nature; (c) central nervous system toxicity resulting in anorexia, lassitude, and nausea; and (d) suppression of reproductive organ function as well as acute vascular effects leading to hypotension and shock.^{2,10,20-22,30,60,63-68}

These mycotoxins are cytotoxic to most eukaryotic cells.^{30,69,70} A number of cytotoxicity assays have been developed that include (a) survival and cloning assays,^{70,71} (b) inhibition of protein^{69,72} and DNA^{73,74} synthesis by radiolabeling procedures, and (c) a neutral red cell viability assay.⁷⁵ It takes a minimum of 24 to 48 hours to measure the effects of trichothecene mycotoxins on cell viability.

Uneo et al⁷⁶ first demonstrated that the trichothecene mycotoxins inhibit protein synthesis in rabbit reticulocytes and ascites cells. The inhibition of protein synthesis by these mycotoxins occurs in a variety of eukaryotic cells.^{59,71,72,77,78} Similar sensitivity to T-2 toxin was observed in established cell lines and primary cell cultures.^{59,72} Protein synthesis inhibition is observed rapidly within 5 minutes after exposure of Vero cells to T-2 toxin, with a maximal response noted within 60 minutes.⁵⁹ A number of studies have concluded that the trichothecene mycotoxins interfere with peptidyl transferase activity and inhibit either the initiation or elongation process of translation.^{77,79-81} Alterations in trichothecene side groups can markedly affect protein synthesis inhibition in in-vitro systems.^{59,70,72,75,77}

Substantial inhibition (86%) of RNA synthesis by trichothecene mycotoxins was observed in human (HeLa) cells,⁷⁷ but T-2 toxin had minor effects (15% inhibition) on RNA synthesis in Vero cells.⁵⁹ In eukaryotic cells, blocking protein synthesis can severely inhibit rRNA synthesis.⁷⁷ Because rRNA accounts for

80% of the total cellular RNA, the trichothecene-mycotoxin-related inhibition of RNA synthesis is probably a secondary effect linked to inhibited protein synthesis.

Scheduled DNA synthesis is strongly inhibited in various cell types exposed to trichothecene mycotoxins.^{59,71,82,83} In mice or rats given a trichothecene mycotoxin, DNA synthesis in all tissues studied was suppressed, although to a lesser degree than protein synthesis.⁸³⁻⁸⁷ Cells require newly synthesized protein to exit G₁ and enter the S phase of the cell cycle,⁸⁸ during which DNA is synthesized. Inhibitors of protein synthesis prevent cells from entering S phase, thereby blocking most DNA synthesis.⁸⁸ Thus, the pattern of DNA synthesis inhibited by the trichothecene mycotoxins is consistent with the primary effect of these toxins on protein synthesis. For the most part, trichothecene mycotoxins do not possess mutagenic activity or the capacity to damage DNA in appropriate cell models.⁵¹

Because the trichothecene mycotoxins are amphiphilic molecules, a number of investigations have focused on various interactions with cellular membranes.^{89,90} Yeast mutants with reduced plasma membrane were more resistant than the parent strain to T-2 toxicity.^{91,92} Changes in cell shape and lytic response to T-2 toxin were observed in studies with erythrocytes, which lack nuclei and protein synthesis.⁹³⁻⁹⁶ Susceptibility to lysis is species dependent and correlates with phosphatidylcholine.⁹⁵ In L-6 myoblasts, uptake of calcium, glucose, leucine, and tyrosine was reduced within 10 minutes after exposure to a low dose of T-2 toxin.⁸⁹ These authors concluded that T-2 exerted multiple effects on the cell membrane.

Once trichothecene mycotoxins cross the plasma membrane barrier, they can interact with a number of targets including ribosomes⁷⁷ and mitochondria.^{92,97-101} These toxins also inhibit electron transport activity, as implied by decreased succinic dehydrogenase activity^{97,100,101} and mitochondrial protein synthesis.⁹⁸ Toxin-stimulated alteration in mitochondrial membranes contributes to the effects on cellular energetics and cytotoxicity. Although initial investigations on the mechanism of action for trichothecene mycotoxins suggested that protein synthesis is the principal target, current observations indicate that the effects of these toxins are much more diverse.

In cell-free or single-cell systems, these mycotoxins rapidly inhibit protein synthesis and polysomal disaggregation.^{10,51,67,102} Thus, it is postulated that the trichothecene mycotoxins can directly react with cellular components. Despite this direct effect, several investigations have been published on the toxicokinetics of the trichothecene mycotoxins.⁵³

Very little of the parent trichothecene mycotoxin is excreted intact; rather, elimination by detoxification is the result of extensive and rapid biotransformation. The biotransformation of T-2 toxin occurs by four competing pathways: (1) ester hydrolysis at the C-4, C-8, and C-15 positions; (2) conjugation with glucuronic acid; (3) aliphatic hydroxylation of the C-3N and C-4N positions on the isovaleryl side chain; and (4) reduction of the 12,13 epoxide.

Clinical Signs and Symptoms of Intoxication

The pathological effects and clinical signs can vary with the route and type of exposure (acute single dose vs chronic subacute doses). Local route-specific effects include the following: (a) dermal exposure leads to local cutaneous necrosis and inflammation^{12,14,103-105}; (b) oral exposure results in upper gastrointestinal tract lesions¹⁰⁶⁻¹⁰⁹; and (c) ocular exposure causes corneal injury.²⁸ For the trichothecene mycotoxins, however, many systemic toxic responses are similar regardless of the exposure route. In contrast, the symptoms and clinical signs of trichothecene intoxication can vary depending on whether the exposure is acute or chronic. For biological warfare use, an acute exposure would be the major concern.

Dermal Exposure

Cutaneous irritations have been observed in individuals exposed to hay or hay dust contaminated with trichothecene-producing molds.³⁵⁻³⁸ While working up large batches of fungal cultures from trichothecene-producing organisms, workers suffered facial inflammation followed by desquamation of the skin and considerable local irritation.¹¹⁰ Applying trichothecene mycotoxins of relatively low toxicity (crotoxin and trichothecin) to the volar surface of a human forearm or to the head resulted in erythema and irritation within a few hours of exposure, followed by inflammation that healed in 1 or 2 weeks.¹¹¹ The hands of two laboratory workers were exposed to crude ethyl acetate extracts containing T-2 toxin (approximately 200 µg/mL) when the extract accidentally got inside their plastic gloves.¹¹¹ Even though the workers thoroughly washed their hands in a mild detergent within 2 minutes of contact, they experienced a burning sensation in their fingers about 4 hours postexposure, which increased in intensity until 8 hours after contact with the toxin. Within 24 hours, the burning sensation had disappeared and was replaced by numbness in the fingers. After about 3 days, sensitivity was lost in all exposed fingers, and by day 4 or 5, the affected skin became hardened and started to turn white. During

the second week, the skin peeled off in large pieces 1 to 2 mm in thickness. By day 18 after contact, normal sensitivity had been regained in the new skin. These observations provide evidence that when human skin is exposed to small amounts of trichothecene mycotoxins, severe cutaneous irritations develop and may last for 1 to 2 weeks after acute exposure. These local skin exposures were too small to cause any detectable systemic reactions.

Several animal models have helped assess the local and systemic toxicity, as well as lethality, from skin exposure to trichothecenes.¹⁴ In a dermal study using a mouse model, T-2 toxin in dimethylsulfoxide was applied to the skin, without the use of a barrier to prevent oral ingestion or removal of the toxin during the grooming process.¹¹² Characteristic radiomimetic effects in the thymus, spleen, and duodenum were easily recognized by 6 hours after topical application of 5 or 40 mg/kg of T-2 toxin.¹¹² Severity of the damage was dependent on the organ evaluated and time after topical exposure. Necrotic skin was present within 6 hours after dermal application of T-2 toxin. With the exception of skin damage, lesions were quantitatively and qualitatively similar to those seen after intragastric application of T-2 toxin. Cumulative mortality and early systemic effects in mice were essentially similar

for topically applied T-2 toxin, HT-2 toxin, DAS, verrucarin A, and roridin A.¹¹³

Regardless of the route of administration, systemic histological lesions associated with T-2 toxin are similar—the most prominent being necrosis of rapidly dividing cells such as those found in the gastrointestinal tract and lymphoid tissues.¹⁴ The severity of necrosis, both local and systemic, is dose dependent. Twenty-four hours after rats were exposed to a dermal dose of 2 mg/kg of T-2 toxin in dimethylsulfoxide, cardiac function was altered, as evidenced by decreased arterial blood pressure, peak intraventricular pressure, and resting systolic and diastolic blood pressure.¹¹⁴ The toxin-treated rats had lower epinephrine-stimulated intraventricular pressure values, indicating reduced contractility. They also exhibited prolonged QT intervals on their electrocardiograms.

Clinical observations and experimental animal studies show that the trichothecene mycotoxins are severe skin irritants (Figure 17-1). If these toxins are applied with absorption enhancers, they cause systemic toxicity at doses comparable to oral or parenteral exposure. Local skin sensitivity and rate of absorption are influenced by a number of factors, including the species, skin thickness and structure, age, nutritional status, and underlying infections.

Ocular Exposure

Ocular exposure may result in tearing, eye pain, conjunctivitis, and blurred vision. A laboratory worker developed burning of the eyes and blurred vision for several days after a powder containing roridin A was accidentally blown into his eyes.⁴³

Cultured filtrates containing roridin A and verrucarin A produced ocular lesions in rabbits.¹⁰⁵ When the filtrates were instilled into the conjunctival sac, erythema and edema of the conjunctival membranes were observed within 1 or 2 days. Later, the cornea became opaque and developed scarring, which persisted as long as 5 months.¹¹⁵ Instillation of trichothecene into the conjunctival sac of a rabbit caused slight inflammation of the conjunctiva, the nictitating membrane, and the eyelids.¹¹⁶ When T-2 toxin (1 μ g) was instilled into the eyes of rats, irregularity of the cornea developed in 12 to 24 hours, which was readily visible with a hand-held ophthalmoscope.^{9,117} Occasionally, corneal staining with fluorescein was positive and diffuse. This lesion would be expected to result in photophobia and decreased acuity. Peak injury was at 24 to 48 hours with recovery in 3 to 7 days. Histologically, this dose of T-2 toxin can cause extreme thinning of the corneal epithelium, which may be irreversible. With exposure

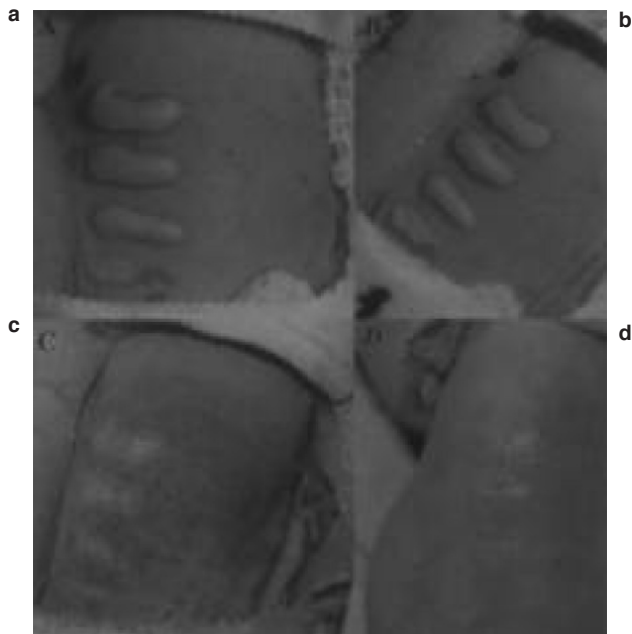


Fig. 17-1. Skin lesions on the back of a hairless guinea pig at (a) 1, (b) 2, (c) 7, and (d) 14 days after application of (bottom to top) 25, 50, 100, and 200 ng of T-2 toxin in 2 μ L of methanol.

to higher doses of T-2 toxin, scleral and conjunctival vasodilatation and inflammation may occur, with corneal irregularities that may persist for 6 months or more.

Because trichothecene mycotoxins can cause severe eye injury that markedly impairs vision, they represent a severe incapacitating problem for unprotected military personnel. No systemic toxicity has been documented from the instillation of trichothecene mycotoxins into the eyes of experimental animals.

Respiratory Exposure

Agricultural workers exposed to hay or hay dust contaminated with trichothecene mycotoxins developed signs and symptoms of upper respiratory injury, including cough, rhinitis, burning in the nose and nasal passages, and nose bleeds.^{35,36} The occupants of a water-damaged house with a heavy infestation of *S atra*, who were exposed to trichothecene-mycotoxin-contaminated dust from the air ducts, complained of a variety of recurring illnesses including cold and flu symptoms, sore throats, diarrhea, headaches, fatigue, dermatitis, intermittent focal alopecia, and general malaise.⁴²

In animal studies, mice, rats, and guinea pigs were exposed to deeply deposited aerosolized T-2 toxin with an average aerodynamic median diameter of 0.6 to 1 μm .²⁰⁻²² At high (lethal) aerosol concentrations of T-2 toxin (2.4 mg/L), mice were lethargic and exhibited no grooming behavior; most were prostrate, and all were dead in 18 hours.²⁰ When exposed to an LD₅₀ aerosol concentration of T-2 toxin (0.24 mg/L), the mice became lethargic and prostrate near death, which occurred in 30 to 48 hours. No significant lesions were observed in the upper respiratory tract or lungs of the exposed mice, rats, or guinea pigs.²⁰⁻²² The microscopic lesions were mainly observed in the lymphoid system and intestinal tract. In a [³H]-labeled T-2 toxin distribution study, approximately 11% and 30% of the total radioactivity was associated with nasal turbinates immediately after a 10-minute exposure of mice with a respective LD₅₀ or LD of aerosolized toxin.²⁰ At the end of this exposure time, only 1% to 2% of the retained radioactivity was found in the respiratory tract; the remainder was distributed throughout the carcass. Thus, approximately 70% to 90% of a retained dose from a 0.6- to 1- μm particle aerosol of T-2 toxin was cleared by the alveoli of the lungs, with a half-life of less than 1 minute. The T-2 toxin associated with the nasal turbinates was probably ingested and may have been responsible for intestinal crypt epithelial necrosis in mice receiving the high-dose aerosol.²⁰

Ingestion

Although aerosol forms of trichothecene mycotoxins are of the most concern as biological warfare weapons, acute ingestion of foods contaminated with large amounts of these mycotoxins could be devastating to soldiers. Chronic subacute ingestion of trichothecene mycotoxins is responsible for atoxic alimentary aleukia, which consists of gastric and intestinal mucosa inflammation that may be followed by leucopenia with progressive lymphocytosis and bleeding diathesis if large amounts are ingested.

Within 4 hours after gastric intubation of a single dose of T-2 toxin, chickens developed asthenia, inappetence, diarrhea, and panting.¹¹⁸ Coma was observed in birds given high doses of T-2 toxin. Death of the birds occurred within 48 hours after T-2 mycotoxin administration. The abdominal cavities of birds given lethal doses contained a white chalk-like material, which covered much of the viscera. Necrosis of the mucosal surface lining the gizzard, as well as thickening, sloughing, and epithelium necrosis in the crop were noted in chickens given a high dose of T-2 toxin. Subacute doses of T-2 toxin resulted in decreased weight gain and feed consumption.

Gastric intubation of an acute dose of T-2 toxin in guinea pigs resulted in lethargy and death within 48 hours.¹¹⁹ Gross lesions included gastric and cecal hyperemia with watery-fluid distension of the cecum and edematous intestinal lymphoid tissue. Histological alterations included necrosis and ulceration of the gastrointestinal tract and necrosis of rapidly dividing cells of bone marrow, lymph nodes, and testes.

Within 20 minutes of a subacute dose of T-2 toxin given by esophageal intubation, a calf developed hind-quarter ataxia, knuckling of the rear feet, listlessness, severe depression, loud teeth grinding, and repeated head submersion in water.¹²⁰ Three days after the initial intubation, the feces became noticeably loose. At necropsy, acute ulceration and necrosis were observed in the gastrointestinal tract.

Parenteral Exposure

The LD₅₀ of T-2 toxin by the intramuscular route in cynomolgus monkeys is 0.75 mg/kg with a 95% confidence limit of 0.4 to 4.2 mg/kg.¹⁴ Similar toxicities were seen for intravenous administration of T-2 toxin in the monkey when administered by a bolus or 4-hour infusion. Mean time to death was 18.4 hours and independent of dose (between 0.65 and 6 mg/kg). Monkeys dosed intramuscularly developed emesis within 30 minutes to 4 hours with doses as low as 0.25

mg/kg.¹⁴ Emesis occurred 15 to 30 minutes after an intravenous dose of T-2 toxin as low as 0.014 mg/kg. The duration and severity of emesis appeared dose-dependent. At 2 to 4 hours postexposure, the monkeys developed a mild to severe diarrhea, especially in the higher dose groups. Listlessness, sluggish response to stimuli, and ataxia occurred 4 to 6 hours postexposure. A progressive hypothermia was evident in dying monkeys. Food intake was reduced in surviving monkeys, even at a dose of 0.014 mg/kg. Severity and duration of food refusal was a function of the toxin dose.

Gross and histological examinations were done on all cynomolgus monkeys that died after exposure to T-2 toxin in various doses. Eight of 16 monkeys showed a mild degree of petechial hemorrhage in the colon and cecum. Three had slight petechial hemorrhages in the small intestine and stomach.¹⁴ Lymphoid necrosis was present in all intoxicated animals. Splenic necrosis was consistently most severe in the white pulp, and lymph node necrosis occurred in the germinal centers, which also affected mature lymphocytes. Gut-associated lymphoid tissue necrosis was a consistent feature ranging from mild to moderate in severity. Thymic necrosis was seen in one of the monkeys, and bone marrow necrosis was observed at higher doses of toxin.¹⁴ Necrosis of glandular elements within the gastrointestinal tract was present in all monkeys, but varied in both severity and distribution, from multifocal to diffuse. The most severe lesions were in the colon. Stomach lesions were inconsistently present in six monkeys. One monkey showed minimal multifocal necrosis of hepatocytes. Seven of the monkeys were diagnosed as having mild nephrosis, consisting of degeneration and necrosis of tubular epithelial cells with no inflammatory response. Heart sections revealed vacuolar change and multifocal degeneration ranging from a mild to moderate degree in eight of the monkeys. One monkey in the high-dose group had a leukoencephalopathy, and three others had minimal focal inflammatory lesions. Multifocal areas of minimal hemorrhage were observed in the spinal cord of four monkeys. Testes from 14 monkeys showed mild multifocal degenerative changes. Minimal to mild hemorrhagic lesions were observed, most commonly in the cecum and heart, in all the monkeys. At doses of T-2 greater than 1 mg/kg, there was minimal hemorrhage in the brain and/or spinal cord. In conclusion, necrosis of lymphoid tissue and glandular epithelium of the gastrointestinal tract were consistent lesions linked to T-2 toxicosis in the monkey. These alterations are also consistent with observations in other species. Among the significant findings was an apparent dose relationship to bone marrow necrosis and leukoencephalopathy, both of

which occurred only in the high-dose groups. Mild lesions in the heart, liver, and kidney are consistent with those observed in other species.^{14,121-125}

Diagnosis

Presumptive Diagnosis

Diagnosis of an attack with trichothecene mycotoxins would largely depend on the clinical observations of casualties and toxin identification in biological or environmental samples, which would involve a combined effort among medical and chemical units in the field. The early signs and symptoms of an aerosol exposure to trichothecene mycotoxins would depend on particle size and toxin concentration. For a large-particle aerosol (particles > 10 μm , found in mist, fog, and dust similar to that allegedly used in Southeast Asia), the signs and symptoms would include rhinorrhea, sore throat, blurred vision, vomiting, diarrhea, skin irritation (burning and itching), and dyspnea. Early signs and symptoms from a deep-respiratory aerosol exposure (from aerosol particles in the 1- to 4- μm range) have not been fully evaluated but could include vomiting, diarrhea, skin irritation, and blurred vision. Later signs and symptoms would probably be similar (except for the degree of skin rash and blisters) for both large-particle and deep-respiratory aerosol exposure to trichothecene mycotoxins. They could include continued nausea and vomiting, diarrhea, burning erythema, skin rash and blisters, confusion, ataxia, chills, fever, hypotension, and bleeding.

Initial diagnostic tests should include standard clinical laboratories and serum, urine, or tissue samples for toxin detection. Nonspecific changes in serum chemistry and hematology occurred in monkeys exposed to an acute dose of T-2 toxin. Alterations in serum chemistries may include elevated serum creatinine, serum enzymes (especially creatine kinase), potassium, phosphorous, and serum amino acid levels. Prothrombin and partial thromboplastin times should also be evaluated by the laboratory because a decrease in coagulation factors may lead to an increased risk of bleeding. An initial rise in the absolute number of neutrophils and lymphocytes may occur within hours, followed by a decrease in lymphocyte counts by 48 hours. Survival beyond several days may be associated with a fall in all blood cellular elements.¹⁴ Although it is likely that these acute changes will be seen in humans, clinical observations among human victims of acute trichothecene mycotoxicosis have not been reported to date. In patients with chronic toxicity resulting from repeated ingestion of contaminated bread, pancytopenia is an important part of the

clinical picture.²⁹ Patients that are exposed to mold and mycotoxins in water-damaged buildings may develop mold-specific immunoglobulin (IgG) and IgE detectable with enzyme-linked immunosorbent assays and radio allegro sorbent test protocols using fungal extracts; however, the elevation of these antibodies has not been statistically associated with morbidity. Secretory IgA against molds and mycotoxins in bronchoalveolar lavage fluid and saliva may be produced in the absence of serum antibodies and may assist in making the proper diagnosis; however, these specific antibodies could be elevated from naturally occurring environmental exposure.

After the yellow rain attacks in Southeast Asia, diagnosis of the causative agent was difficult and involved ruling out conventional chemical warfare agents. An attack with mycotoxins alone would not contaminate the environment and clothing with nerve and blistering agents, and these agents were not detectable in such samples from Southeast Asia. The following events should suggest that a biological warfare attack with trichothecene mycotoxins has occurred: (a) clinical findings that match the symptoms listed above; (b) high attack and fatality rates; (c) dead animals of various types in the attack area; and (d) onset of symptoms after a yellow rain or red, green, or white smoke or vapor attack.

Several commercial immunoassay kits are marketed for detecting trichothecene mycotoxins (T-2 toxin, deoxynivalenol, and their metabolites) in grain extracts or culture filtrates of *Fusarium* species.^{126,127} The US Department of Agriculture has published a manuscript by the Grain Inspection, Packers and Stockyards Administration Technical Services Division that lists approved tests for this use; however, these kits have not been evaluated against biomedical samples that contain typical concentrations of the mycotoxins. Screening tests for presumptive identification of trichothecene mycotoxins in the biomedical samples would involve bioassays, thin-layer chromatography (TLC), or immunological assays, in any combination. At a national laboratory, confirmatory methods involve the use of various techniques that include gas chromatography, high-performance liquid chromatography (HPLC), mass spectrometry (MS), and nuclear magnetic resonance spectrometry.

In areas that have experienced a yellow rain attack, environmental assays have been in the range of 1 to 150 parts per million (ppm) and blood samples in the range of 1 to 296 parts per billion (ppb).^{1,128} Ten and 50 minutes after an intramuscular injection of 0.4 mg/kg of T-2 toxin in dogs, plasma concentrations of T-2 toxin were respectively 150 and 25 ppb, and 50 and 75 ppb for HT-2 toxin.¹²⁹ Thus, any screening procedure

for trichothecene mycotoxins in biomedical samples must have detection limits of 1 to 100 ppb. Most of the analytical procedures require extraction and cleanup treatments to remove interfering substances.

Screening tests for the trichothecene mycotoxins are generally simple and rapid but, with the exception of the immunochemical methods, are nonspecific. Several bioassay systems have been used to identify trichothecene mycotoxins. Although most of these systems are very simple, they are not specific, sensitivity is relatively low compared to other methods, and they require that the laboratory maintain vertebrates, invertebrates, plants, or cell cultures. TLC is one of the simplest and earliest analytical methods developed for mycotoxin analysis. Detection limits for trichothecene mycotoxins by TLC is 0.2 to 5 ppm (0.2 to 5 $\mu\text{g}/\text{mL}$). Therefore, extracts from biomedical samples would have to be concentrated 10-fold to 1,000-fold to screen for trichothecene mycotoxins.

To overcome the difficulties encountered with the bioassays and TLC methods, immunoassays using specific polyclonal and monoclonal antibodies have been developed for most of the major trichothecene mycotoxins and their metabolites. These antibodies have been used to produce simple, sensitive, and specific radioimmunoassays and enzyme-linked immunosorbent assays for the mycotoxins. The lower detection limit for identification of trichothecene mycotoxins by radioimmunoassay is about 2 to 5 ppb,¹³⁰ and by enzyme-linked immunosorbent assay, 1 ppb.¹³¹

Confirmatory Procedures

Gas-liquid chromatography (GLC) and HPLC are two of the most commonly used methods for identifying trichothecene mycotoxins in both agricultural products and biomedical samples; however, extensive treatment to clean up the sample is required before derivatization and subsequent analysis. By the most sensitive procedures, detection limits for trichothecene mycotoxins is 10 ppb. If the analysis is on a sample that contains an unknown toxic material, such as that from a yellow rain attack, then the GLC method can provide only presumptive evidence of a trichothecene mycotoxin exposure. Confirmation requires identification with more definitive physicochemical procedures.

MS is the physicochemical method of choice for characterizing, identifying, and confirming the presence of trichothecene mycotoxins. Picogram quantities of trichothecene mycotoxins are readily detectable by MS methods. In some cases, extensive cleanup steps are unnecessary. The combination of GLC and MS techniques (GLC-MS) has proven to be a more specific method for identifying mycotoxins than GLC

alone,^{132,133} and it has become the standard for identifying trichothecene mycotoxins in agricultural products and biomedical samples. As little as 1 ppb of T-2 toxin can be identified without extensive cleanup¹³²; however, the method requires a time-consuming derivatization step. A high-performance liquid chromatography–mass spectrometry (HPLC–MS) procedure, described in 1991, provides a specific, reliable method for identifying trichothecene mycotoxins without derivatization,¹³⁴ achieving sensitivity at the 0.1-ppb level. HPLC-MS and GLC-MS are the best and most sensitive methods for detecting mycotoxins. Additionally, HPLC-MS can be used with diode array detection (DAD), which measures the ultraviolet spectrum of a sample. HPLC-DAD-MS limits of detection range from 1 pg to 3 ng.

Medical Management

Preexposure Treatment and Decontamination

The immediate use of protective clothing and mask at the first sign of a yellow-rain–like attack should protect an individual from the lethal effects of this mycotoxin. Because the area covered with agent is likely to be small, another helpful tactic is to simply leave the area. A lightweight face mask, outfitted with filters that block the penetration of aerosol particles 3 to 4 μm or larger, should provide respiratory protection against yellow rain. Only 1% to 2% of aerosolized T-2 toxin penetrated nuclear, biological, and chemical protective covers.¹³⁵ Regular military uniforms would offer some protection, depending on the age and condition of the fabric as well as the environmental conditions.

Skin exposure reduction paste against chemical warfare agents (SERPACWA), a Food and Drug Administration–approved preexposure skin treatment for use against chemical warfare agents and dermally active toxins, functions by forming a physical barrier on the skin. SERPACWA is designed for application at closure points of chemical over-garments—the neck, wrists, and ankles—as well as sweat-prone areas such as the armpits and groin. When SERPACWA was applied to anesthetized rabbits that were then exposed to a 6-hour challenge with T-2 mycotoxin, all signs of dermal irritation were blocked for 24 to 48 hours. However, SERPACWA must be applied before an attack; it is not effective after exposure.

As soon as individuals or units suspect exposure to a mycotoxin attack, they should remove their uniform, wash their contaminated skin with soap and water, and then rinse with water. Washing the contaminated skin area within 4 to 6 hours after exposure to T-2 toxin removes 80% to 98% of the toxin, thus prevent-

ing dermal lesions and death in laboratory animals.¹³ Contaminated uniforms as well as wash waste from personnel decontamination should be exposed to household bleach (5% sodium hypochlorite) for 6 hours or more to inactivate any residual mycotoxin. The M291 decontamination kit for skin contains an XE-555 resin material as the active component, which is efficacious against most chemical warfare agents and presents no serious human safety problems. The XE-556 resin, a similar but different formulation, was effective in the physical removal of T-2 toxin from the skin of rabbits and guinea pigs.¹³⁶ The foregoing observations suggest that skin decontamination kits designed specifically for detoxification of chemical warfare agents could also provide protection by physically removing mycotoxins from the skin of exposed individuals.

Specific and Supportive Therapy

No specific therapy for trichothecene-induced mycotoxicosis is known or is presently under experimental evaluation. Several therapeutic approaches have been evaluated in animal models. Although experimental procedures for treating systemic exposure have successfully reduced mortality in animal models, they have not been tested in primates, and they are not available for field use in humans potentially exposed to trichothecene mycotoxins.

Individuals exposed to a yellow-rain–like attack should be treated with standardized clinical toxicology and emergency medicine practices for ingestion of toxic compounds. After an aerosol exposure, mycotoxins will be trapped in the nose, throat, and upper respiratory tract. The particles will be swallowed via ciliary action, resulting in a significant oral exposure. Superactive charcoal has a very high maximal binding capacity (0.48 mg of T-2 toxin per mg of charcoal), and treatment either immediately or 1 hour after oral or parenteral exposure to T-2 toxin significantly improves the survival of mice.¹³⁷

Symptomatic measures for treating those exposed to trichothecene mycotoxins are modeled after casualty care for mustard poisoning. Irrigation of the eyes with large volumes of isotonic saline may assist in mechanically removing trichothecene mycotoxins, but such treatment would have limited useful therapeutic effects. Casualties with ocular involvement will likely need detailed ophthalmologic evaluation for corneal lesions and treatment to prevent vision loss, secondary infection, and the development of posterior synechie. After the skin has been decontaminated, some erythema may appear and accompany burning and itching sensations. Most casualties whose skin has

been treated with soap and water within 12 hours of exposure will have mild dermal effects, which can be relieved by calamine and other lotions or creams.

Limited data are available on the respiratory effects of inhaled trichothecene mycotoxins, although acute pulmonary edema was one of the serious, often lethal, consequences of a yellow rain attack. One of the major symptoms after the yellow rain attacks was an upper respiratory irritation consisting of sore throat, hoarseness, and nonproductive cough, which may be relieved by steam inhalation, codeine, cough suppressants, and other simple measures. A casualty who develops severe respiratory symptoms may require endotracheal intubation with positive pressure ventilation to maintain airway patency and oxygenation. A physician trained in pulmonary or intensive care medicine should conduct any required advanced airway management, with a focus upon maintaining ventilation and oxygenation, as well as preventing secondary infection. Theoretically, granulocyte-stimulating factors may be useful for patients who develop bone marrow suppression.

The early use of high doses of systemic glucocorticosteroids increases survival time by decreasing the primary injury and shock-like state that follows exposure to trichothecene mycotoxins.¹³⁸ Additionally, dosing before and after the exposure with diphenhydramine (an antihistamine) or naloxone (an opioid antagonist) prolonged the survival times of mice exposed subcutaneously or topically with lethal doses of T-2 toxin.¹³⁹

Several bioregulators might mediate the shock-like state of trichothecene mycotoxicosis. Methylthiazolidine-4-carboxylate increased hepatic glutathione content and enhanced mouse survival after an acute intraperitoneal exposure to T-2 toxin.¹⁴⁰ The protective effects of this drug may result from increased detoxification and excretion of the glucuronide conjugate of T-2 toxin. A general therapeutic protocol that included combinations of metoclopramide, activated charcoal, magnesium sulfate, dexamethasone, sodium phosphate (which had very little effect), sodium bicarbonate, and normal saline was evaluated in swine given an intravenous LD₅₀ dose of T-2 toxin.¹⁴¹ All treatment groups showed improved survival times compared to survival of the nontreated controls.

Prophylaxis

To date, there is no licensed vaccine to protect against the mycotoxins. The mycotoxins are low-molecular-weight compounds that must be conjugated to a carrier protein to produce an effective antigen.¹³⁰ When T-2 toxin is conjugated to a protein, it elicits relatively low antibody titers and remains a marked skin irritant.¹⁴² This would preclude the use of mycotoxins as immunogens in eliciting protective immunity. To circumvent such problems, a deoxy-verrucarol-protein conjugate was used to vaccinate rabbits.¹⁴³ Antibody titers developed rapidly after vaccination, but they were highly specific for the conjugate rather than for a common trichothecene backbone.

Another approach was to develop antibody-based (antiidiotypic) vaccines against T-2 toxin. Protective monoclonal antibodies were generated and used to induce specific monoclonal antiidiotypic antibodies. When mice were vaccinated with these antibodies, they developed neutralizing titers that protected against challenge with a lethal dose of T-2 toxin.¹⁴⁴ Thus, an antiidiotypic antibody would be feasible as a vaccine candidate against T-2 toxin.

Several monoclonal antibodies against T-2 toxin will protect against the T-2-induced cytotoxicity in various cell lines. When a monoclonal antibody against T-2 toxin (15H6) was given to rats (250 mg/kg) 30 minutes before or 15 minutes after a lethal dose of mycotoxin, it protected 100% of them.¹⁴⁵ Thus, monoclonal antibodies do have some prophylactic and therapeutic value against T-2 toxicosis, but very large quantities are required for protection.

Prophylactic induction of enzymes involved in conjugating xenobiotics reduced or prevented the acute toxic effects of T-2 toxin in rats, whereas inhibition of these enzymes resulted in a higher toxicity.¹⁴⁶ Pretreatment with flavonoids, ascorbic acid, vitamin E, selenium, or chemoprotective compounds such as emetine that block trichothecene-cell association all reduce acute toxicity of these mycotoxins. However, none of these chemoprotective treatments has undergone extensive efficacy studies to evaluate their ability to protect against an aerosol or dermal exposure to trichothecene mycotoxins.

MARINE ALGAL TOXINS

History

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, and others have been implicated in mass mortalities of birds and marine mammals. However, their presence in the environment is more often "silent," detectable only when contaminated foodstuffs are ingested. The long-term environmental and public health effects of

chronic exposure in humans are poorly understood, although questions are beginning to arise about whether chronic exposures to some marine toxins may increase the risk of cancer through their action as tumor promoters.

Ingesting seafood contaminated with marine biotoxins can cause six identifiable syndromes: (1) paralytic shellfish poisoning (PSP), (2) neurotoxic shellfish poisoning (NSP), (3) ciguatera fish poisoning, (4) diarrhetic shellfish poisoning, (5) amnesic shellfish poisoning (ASP), and (6) azaspiracid poisoning. With the exception of ciguatera fish poisoning, which, as the name implies, is caused by eating contaminated finfish, all are caused by ingesting shellfish. With the exception of ASP, which is of diatom origin, the causative toxins all originate from marine dinoflagellates.

The toxin-producing algal species are a small fraction of the thousands of known phytoplankton. 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 many 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 the development of traditional biological warfare weaponry.

Targeting food supplies as an act of biological terrorism is a much 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. This negates the requirement 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 inserted into the food supply at the desired location. Regulatory testing, if any, is typically done only at the harvester and distributor levels.

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 low. In other cases, harvesting could be easy. The United States and other

countries maintain monitoring programs at the state and local level 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 that time.

Of the six marine toxin syndromes, three—ciguatera fish poisoning, diarrhetic shellfish poisoning, and azaspiracid poisoning—are unlikely to be a significant bioterrorism threat. 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 kind of turmoil sought by terrorists. Ciguatera fish poisoning can present a much more serious intoxication, but toxic fish are extremely difficult to procure. Acquiring sufficient material to launch a food-related bioterrorist attack of any magnitude is nearly impossible.

The three marine algal toxin syndromes with bioterrorism potential and the causative toxins (Table 17-1) are described in the following section. Some are a 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 heterocyclic guanidines called paralytic shellfish poisons, or gonyautoxins. STX was the first known member of this family, named for the giant butter clam, *Saxidoma giganteus*, from which it was first isolated.¹⁴⁷ Later it was learned that STX is the parent compound of over 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. More recently, STX was isolated from bacterial species associated with dinoflagellate cells, suggesting the possibility of a bacterial origin for at least some dinoflagellates.¹⁴⁸ 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.¹⁴⁹

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, Europe, Japan, Australia, Southeast Asia, and India. The overall mortality rate has been estimated at 15%,¹⁵⁰ although mortality is highly dependent upon the quality of medical care received.

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 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.

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. The most common symptom in these outbreaks was parasthesia, 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.¹⁵¹

Approximately 10 outbreak-associated PSP cases are reported to the Centers for Disease Control and Prevention each year. In 2002 there were 13 cases of neurological illness associated with consumption of pufferfish containing STX caught near Titusville, Florida.¹⁵² All 13 symptomatic patients reported tingling or numbness in the mouth or lips. Additionally, eight reported numbness or tingling of the face, ten

TABLE 17-1
COMPARISON OF SELECTED MARINE ALGAL TOXINS

	Paralytic Shellfish Poisoning	Neurotoxic Shellfish Poisoning	Amnesic Shellfish Poisoning
Toxin	Gonyautoxins (saxitoxin)	Brevetoxins	Domoic acid
Source	Marine dinoflagellates	<i>Karenia brevis</i>	<i>Pseudo-nitzschia multiseries</i>
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

reported these symptoms in the arms, seven reported these symptoms in the legs, and one 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.¹⁵³

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.¹⁴⁴ 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.^{154,155}

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.¹⁵⁵

Inhalation. In mice, STX is significantly more toxic by inhalation (LD_{50} of 2 $\mu\text{g}/\text{kg}$) or by intraperitoneal injection (LD_{50} of 10 $\mu\text{g}/\text{kg}$) than by oral administration (LD_{50} of 400 $\mu\text{g}/\text{kg}$).¹⁵⁶ Unlike PSP in humans, which is an oral intoxication and has a lag time to toxicity resulting from absorption 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.^{157,158} 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.¹⁵⁹ Intoxication with large doses of STX may lead to metabolic acidosis, cardiac dysrhythmias, and cardiogenic shock, even with correction of ventilatory failure.¹⁶⁰

Diagnosis

Clinicians should consider PSP in patients who present with rapid onset of neurological symptoms that are sensory, cerebellar, and motor in nature and occur shortly after consumption of seafood.

Confirmatory diagnosis should rely on analysis of body fluid samples, including serum and urine, as well as analysis of gastric contents or uneaten portions of recent meals. Animal studies have demonstrated that STX is excreted primarily in urine. After intravenous injection of STX in rats, 19% of the toxin was excreted 4 hours after injection. By 24 hours, 58% of the toxin was excreted, but small quantities of unmetabolized STX were still detected up to 144 hours after administration.

Postmortem examinations of fatally intoxicated humans have identified STX in gastric contents; body fluids including serum, urine, bile, and cerebrospinal fluid; and tissues including the liver, kidneys, lungs, stomach, spleen, heart, brain, adrenal glands, pancreas, and thyroid.^{157,158} The largest concentrations of STX were 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. HPLC can detect individual toxins but requires either precolumn or postcolumn derivatization of toxin mixtures for optimal detection.^{161,162} Receptor-binding assays based on either rat brain membranes¹⁶³ or purified STX-binding proteins from frogs or snakes¹⁶⁴ measure total biological activity regardless of toxin profile. All of these have been used to detect paralytic shellfish poisons in the urine and serum of intoxicated victims.¹⁵⁵ Antibody-based assays can detect major toxins, but cross-reactivity among minor paralytic shellfish poisons is highly variable. Rapid-test kits are now commercially available.

Medical Management

Treatment for STX intoxication is supportive care. Patients who have recently ingested the toxin may benefit from gastric lavage to expedite removal of the

toxin from the gastrointestinal tract. Patients need to be monitored closely for at least 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. Intoxication with 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 severe metabolic acidosis. Vasopressor agents should 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 to cats intravenously, dobutamine improved recovery over dopamine.¹⁶⁰

There is no specific therapy for patients with STX intoxication. Research into specific therapies has included use of anti-STX serum and antibodies as antidotes, and the use of pharmacologic agents to overcome inhibition of the voltage-dependent sodium channel.

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 organisms occur annually on 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. 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 and 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 1844.¹⁶⁵ 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 breve-

toxins.¹⁶⁶ 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, in 1993 an outbreak was reported in New Zealand. In 2000 blooms of another dinoflagellate, *Chattonella verruculosa*, occurred in Rehoboth Beach, Delaware, and caused a series of localized fish kills.¹⁶⁷ 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.¹⁶⁸ 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 potentials¹⁶⁹ and inappropriate ionic flux. Clinical effects are typically more centrally mediated than peripherally mediated.

Brevetoxin 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 receptor, and excitotoxic cell death.¹⁷⁰ A detailed review of the molecular pharmacology and toxicokinetics of brevetoxin can be found in Poli's *Recent Advances in Marine Biotechnology, Volume 7: Seafood Safety and Human Health*.¹⁷¹

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.¹⁷² Although a fatal case of NSP has never been reported, children may be more susceptible, and a fatal dose must be considered a possibility.¹⁶⁶

The toxic dose of brevetoxins in humans has not been established. However, important information has recently been gleaned from a clinical outbreak. In 1996 a father and two small children became ill after ingesting shellfish harvested in Sarasota Bay, Florida. 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 postingestion.¹⁶⁶ 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) were unknown.

The toxicity of brevetoxins in mice is well established. LD₅₀ 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 6600 µg/kg for PbTx-3 and PbTx-2, respectively.¹⁷³ 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.¹⁷⁴

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. 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.¹⁷⁵

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.¹⁷⁶

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 the young, the elderly, 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

There is no specific therapy 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 IgG showed that treatment before exposure blocked nearly all neurological symptoms.¹⁷⁷ 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.^{178,179} 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. Over 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.¹⁸⁰ Domoic acid was previously known as a compound tested and rejected as a potential insecticide and is a common ingredient in Japanese rural folk medicine. Domoic acid was originally isolated from a red alga, and researchers were surprised to discover that the diatom *Pseudo-nitzschia pungens f multiseriis* (now *Pseudo-nitzschia multiseriis*) was its causative organism. ASP remains the first and only known seafood toxin produced by a diatom.

Since the 1987 outbreak, toxic species of *Pseudo-nitzschia* 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 *P 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.¹⁸¹ In 1998 over 400 sea lions died and numerous others became ill after ingesting anchovies feeding in a bloom of *P australis*, again in Monterey Bay.¹⁸² Domoic acid was detected in both the anchovies and feces from the sea lions.¹⁸³ 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 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.¹⁸⁴ This causes a protracted influx of cations into the neurons and stimulates a variety of intracellular events leading to cell death.¹⁸⁵ This effect may be potentiated by synergism with the excitotoxic effects from high glutamate and aspartate levels found naturally in mussel tissue.¹⁸⁶ The kainate and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid 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.¹⁸⁷ Neuropathological studies of four human fatalities revealed neuronal necrosis or loss with astrocytosis, mainly affecting the hippocampus and the amygdaloid nucleus.¹⁸⁸

Clinical Signs and Symptoms

Ingestion. The 1987 Prince Edward Island outbreak provided information on the clinical effects of domoic acid ingestion in humans.¹⁸⁹ The outbreak occurred during November and December, with 250 reports of illness related to mussel consumption (107 of these

reports met 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.¹⁸⁹ Severe neurological manifestations, more common in the elderly, 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 in the average range 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.¹⁸⁸ 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.¹⁸⁹

Symptoms of intoxication occur after a latency period of a few hours. In the outbreak's 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.¹⁸⁹ In a study of 14 patients who developed severe neurological manifestations, 13 developed gastrointestinal symptoms between 1 and 10 hours after ingestion of seafood, 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.¹⁸⁸ All the patients who developed severe neurological symptoms were older than 65 or had pre-existing medical conditions such as diabetes or renal failure that altered their renal clearance.

Inhalation. There are no natural cases of domoic acid inhalation, 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 that of other exposure routes, although no data are available to confirm this theory.

Diagnosis

Diagnosis should be suspected by the clinical presentation after ingestion of a seafood meal. 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 over the age of 65 or have underlying medical conditions that affect renal clearance.¹⁸⁹ 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 that may be used to rule out other clinical causes of the symptoms include imaging with computed tomography scans, which do not show abnormality related to domoic acid intoxication, and monitoring of brain activity with electroencephalogram. 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.¹⁸⁹ 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.¹⁸⁸

Based primarily on levels measured in Canadian shellfish after the 1987 outbreak, it is thought that mild symptoms in humans might appear after ingestion of approximately 1 mg/kg of domoic acid, and severe symptoms may follow ingestion of 2 to 4 mg/kg. The current regulatory limit for shellfish in Canada, the United States, and the European Union is 20 µg/g, although the European Union is revising this downward. The official regulatory testing method uses analytical HPLC, although both immunological methods and a simple, inexpensive TLC method are available.¹⁹⁰⁻¹⁹² There is no evidence of domoic acid metabolism by rodents or primates, as shown by recovery in an unchanged form from the urine or feces.¹⁹³ 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 electroencephalogram.¹⁹⁴

There is no specific therapy for domoic acid intoxications. Research has revealed that competitive and noncompetitive N-methyl-D-aspartate receptor antagonists reduce the excitable amino acid cascade that leads to brain lesions.¹⁷⁰ Additionally, non-N-methyl-D-aspartate receptor antagonists have also been shown to antagonize domoic acid toxicity.¹⁹⁵

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.

CLOSTRIDIAL TOXINS

History

Clostridium perfringens is a gram-positive, spore-forming anaerobe commonly found throughout nature (eg, in soil, water, and the gastrointestinal tract). It is regarded as one of the most toxic bacteria known, with 17 different protein toxins described to date.¹⁹⁶ However, unlike several other bacterial pathogens (eg, *Listeria*, *Rickettsia*, *Salmonella*, *Shigella*, and *Yersinia* species), *C perfringens* pathogenesis is not generally thought to involve invasion of, and replication in, eukaryotic cells. By using technologies first developed in Robert Koch's laboratory at the Hygiene Institute of Berlin, William Welch and George Nuttall discovered the bacterium in 1892 at Johns Hopkins University in Baltimore. *C perfringens* has also been known in the literature as *Bacillus aerogenes capsulatus*, *Bacillus welchii*, or *Clostridium welchii*.

C perfringens consists of five toxin types (A, B, C, D, and E) as shown in Table 17-2, based upon the production of four major toxins (alpha, beta, epsilon, and iota). These toxins are lethal, dermonecrotic, and associated with a wide range of diseases and intoxications,

including a rapid, life-threatening myonecrosis (gas gangrene) and various animal and human enterotoxemias (Table 17-3).

A major form of human food poisoning found worldwide is caused by another protein toxin, *C perfringens* enterotoxin, which is naturally synthesized during bacterial sporulation in the small intestine following ingestion of *C perfringens* in tainted food. Type A strains are most prevalent in the environment and most commonly linked with human disease. *C perfringens* (namely type A) has historically had a huge impact on those wounded during combat. Gangrene

TABLE 17-2
THE MAJOR TOXIN TYPES OF CLOSTRIDIUM PERFRINGENS

Toxin	A	B	C	D	E
Alpha	x	x	x	x	x
Beta		x	x		
Epsilon		x		x	
Iota					x

TABLE 17-3
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 (pigbel, darmbrand, or "fire-belly"), pigs, calves, goats, and foals Enterotoxemia in sheep (struck)
D	Enterotoxemia in lambs (pulpy kidney disease) and calves Enterocolitis in goats and cattle
E	Cattle and dog enteritis

from *C perfringens* (also known as clostridial myonecrosis) and other anaerobes resulting from wound contamination in the field or in nonsterile operating theaters (particularly prevalent before 1900) resulted in many amputations and deaths that would be unlikely to occur today. If administered soon after infection and the onset of disease, surgical debridement, various antibiotics (eg, beta-lactams, clindamycin, and metronidazole), and hyperbaric oxygen provide effective treatments for most cases of gangrene induced by *C perfringens*.

Protein toxins, considered the major virulence factors for *C perfringens*, have received considerable attention by various laboratories throughout the world. For example, progression of *C perfringens*-induced gangrene is linked to the alpha toxin (a zinc-dependent phospholipase C), which has profound effects upon endothelial cells, including (a) production of proinflammatory compounds; (b) aberrant binding of polymorphonuclear cells to endothelial cells in blood vessels around, but not in, the site of myonecrosis; and (c) enhanced vascular permeability.^{197,198} Specific antibodies against alpha toxin have proven efficacious in preventing gangrene, as demonstrated by recent vaccination studies in a mouse model.¹⁹⁹ For many pathogens, toxins play important roles in survival, such as obtaining nutrients and thwarting the host's immune system. There are two primary modes of action described for the four major toxins produced by *C perfringens*: (1) "punching" holes in cell membranes (alpha, beta, and epsilon toxins), which causes ion imbalances and general leakiness; and (2) disruption of the actin cytoskeleton (iota toxin). In either scenario, the end result is the same: cell death. Studies of *C perfringens* from many laboratories show that the microorganism has evolved effective offensive (toxins) and defensive (toxins and spores) tools for surviving and thriving in diverse environments.

Because of recent national and international biodefense concerns, the epsilon toxin has been considered a potential problem for both civilians and the military.²⁰⁰ As determined by LD₅₀, epsilon is the most potent of all *C perfringens* toxins, and ranks behind only the *C botulinum* and *C tetani* neurotoxins among all clostridial toxins. The Centers for Disease Control and Prevention have placed epsilon toxin on the category B list of select agents, along with bacterial diseases (eg, brucellosis, glanders, and typhus) and other protein toxins (eg, ricin, staphylococcal enterotoxin B). Epsilon toxin represents a potential agroterrorism threat, and is thus also deemed a select agent by the US Department of Agriculture (<http://www.cdc.gov/od/sap/docs/salist.pdf>).

Description of the Epsilon Toxin

Natural Occurrence

Naturally, epsilon toxin is produced by type B and D strains of *C perfringens* involved in animal (eg, cattle, goats, and sheep) enterotoxemias, which are often widespread, rapidly fatal, and economically damaging for the agriculture industry. Although *C perfringens* is considered normal intestinal flora in ruminants, types B and D cause life-threatening problems if introduced, respectively, into the digestive system in newborn animals or, after a diet change to higher carbohydrate levels (in particular starch), in older animals.¹⁹⁶ When there is little microbial competition, or a richer diet suddenly becomes available, resident *C perfringens* types B and D can rapidly proliferate in the intestines and produce a number of toxins, including epsilon. Epsilon toxin and *C perfringens* types B and D infections are linked to veterinary rather than human disease, which establishes an unusual scenario in the event of its use as a biological weapon against humans (possibly advantageous to the perpetrator). In such a situation, physicians would have difficulty diagnosing the resulting unusual syndrome. The following explanation of the biochemistry and biology of epsilon toxin in animals may provide useful information for a potential incident of epsilon intoxication within the general human population.

Chemical and Physical Properties

C perfringens epsilon toxin is synthesized from plasmid DNA as a 311-amino-acid "protoxin" that is subsequently activated extracellularly by proteolytic removal of small peptides at both the amino-terminal (13 residues) and carboxy-terminal (22 residues). In this sense, the toxin is resistant to inactivation by serine-type proteases commonly found throughout nature. The protoxin also contains a typical leader sequence (32 amino-terminal residues) that facilitates secretion from the bacterium into the environment. The crystal structure (Figure 17-2) reveals three domains and a shared conformation with another pore-forming toxin, aerolysin. Aerolysin is produced by *Aeromonas hydrophila* strains associated with ulcerative fish disease.²⁰¹ Proteolytic loss of the carboxy-terminus from epsilon toxin seems primarily responsible for activation and subsequent homoheptamer formation.²⁰² In epsilon toxin, proteolysis, a common method of activating bacterial toxins, induces conformational changes that facilitate oligomerization on the cell surface. In essence, proteolytic activation is a "protein priming" event that enables the protein toxin to act quickly after binding to a cell. Additionally, proteolysis of the amino-terminal

and carboxy-terminal on the epsilon protoxin leads to a more acidic isoelectric point, which may play a role in receptor interactions.²⁰³ For enteric-produced toxins requiring proteolysis, the proteases synthesized by resident bacteria²⁰⁴ and host²⁰² are bountiful.

Mechanism of Action

The mode of action for epsilon toxin involves pore formation in cell membranes facilitated by detergent-resistant membrane fractions (also known as lipid rafts) that concentrate toxin monomers into homoheptamers.^{205,206} Epsilon toxin oligomers formed at 37°C are more stable than oligomers formed at 4°C, as shown by analysis of samples treated with detergent (sodium dodecyl sulfate) and heat before polyacrylamide gel electrophoresis.²⁰⁷ Recent research suggests that these dynamic, cholesterol-rich membrane domains play important roles in many diseases elicited by bacteria (and associated toxins) and viruses.²⁰⁸ Although largely unexplored, the burgeoning field of lipid rafts is apparently fertile for future therapeutic endeavors. Secondary effects of epsilon toxin involve cytoskeletal disruption,²⁰⁹ which, in concert with the disrupted membrane integrity facilitating free passage of 1 kDa molecules,²¹⁰ inevitably proves lethal for an intoxicated cell. Additionally, the integrity of cell monolayers is readily disrupted by epsilon toxin,²⁰⁵ which provides another clue to understanding edema involving the blood-brain barrier.²¹¹

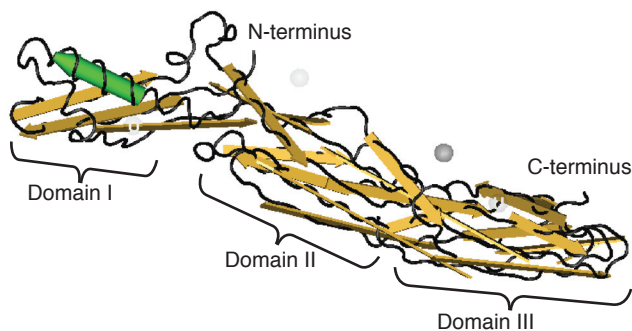


Fig. 17-2. Crystal structure of *Clostridium perfringens* epsilon protoxin. Based on analogous regions on other pore-forming toxins such as *Aeromonas hydrophila* aerolysin, there are three domains putatively involved in receptor binding (domain I), oligomerization (domain II), and membrane insertion (domain III).

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) Chen J, Anderson JB, DeWeese-Scott C, et al. MMDb: Entrez’s 3D-structure database. *Nucleic Acids Res.* 2003;31:474–477.

Clinical Signs and Symptoms

Although epsilon toxin is readily found in the heart, lungs, liver, and stomach following intoxication, it noticeably accumulates in kidneys, causing what veterinarians call “pulpy kidney disease.”^{196,212–214} Toxin accumulating in the kidney may represent a natural defense mechanism by the host to prevent lethal toxin concentrations in the brain.^{214,215} The neurotropic and lethal aspects of *C perfringens* epsilon toxin are of utmost concern²¹² (contributing to the toxin’s listing as a category B select agent). Among neuronal cell populations, the neurons are most susceptible, followed by oligodendrocytes and astrocytes.²¹⁶ These neurotropic aspects cause profound effects in animals that succumb naturally to epsilon-toxin-producing *C perfringens*. Experimentally, the clinical signs attributed to epsilon toxin given intravenously to calves, lambs, and young goats occurred very quickly (in approximately 30 minutes).^{217,218} The animals experienced labored breathing, excited or exaggerated movements, intermittent convulsions, loss of consciousness, and ultimately death. Results from another laboratory revealed that an intravenous injection of epsilon toxin (2–4 LD₅₀) into mice also yields seizures within 60 minutes. The intravenous LD₅₀ for epsilon toxin in mice is low, at approximately 70 ng/kg.²¹⁵ Duodenal inoculation of goats with whole culture or supernatant of *C perfringens* type D led to diarrhea, respiratory distress, and central nervous system dysfunction (ie, recumbency and convulsions).²¹⁹ Similar symptoms were also evident in lambs, except for the diarrhea.²²⁰ The mode of action for epsilon toxin in vivo likely involves ion imbalance, endothelial disruption, and edema. *C perfringens* epsilon toxin establishes a vicious cycle in the gut, with increased permeability of the intestinal tract leading to higher circulating levels of toxin.²¹⁶ It is clear in different animal models that the toxin is active when given intravenously or intraduodenally; however, the literature contains no data on either oral or aerosol routes of intoxication for epsilon toxin.

Medical Management

Partly because of its natural association with animal rather than human disease, there has been little study of therapy for *C perfringens* epsilon toxin. An effective vaccine against epsilon toxin (described below) is readily available for animal use, thus obviating the need for a therapeutic in susceptible animal populations. No therapeutic treatment or vaccine against epsilon toxin has been approved for human use. However, two studies, one in vivo and the other in vitro, suggest that therapy might be possible. One

endeavor by Miyamoto et al²¹⁵ showed that riluzole, a drug that prevents presynaptic glutamate release used for treating human amyotrophic lateral sclerosis, can minimize murine seizures induced by epsilon toxin. However, these results were derived from an injection of riluzole given 30 minutes before toxin, and the drug was evidently not used in subsequent experiments as a therapeutic (ie, administered after toxin injection).

The in-vitro study, recently reported by Beal et al,²²¹ showed that tolerance toward epsilon toxin occurs in various cell lines, especially Madin-Darby canine kidney cells, when incubated with increasing amounts of toxin over time. Concomitantly, a group of unknown acidic proteins was lost (or possibly shifted to a different isoelectric point) from the cells that become tolerant to epsilon toxin (vs untreated controls). Exactly how this mechanism works and how such findings can be exploited as a therapy are still unresolved.²²¹ Similar results with increased cell resistance (although possibly involving another mechanism) to the lethal toxin produced by *Bacillus anthracis*, the causative agent of anthrax, have also been discovered.²²²

Additional therapy and prophylaxis studies show that the epsilon protoxin affords protection (delayed time to death) in mice 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.²¹² In 1976 Buxton²²³ discovered that a formalin toxoid of the protoxin affords protection (up to 100 minutes) after epsilon toxin exposure. Such data suggest that a receptor-targeted approach for prophylaxis is possible, and that a receptor antagonist (ie, receptor-binding domain or small molecular weight competitor) may be useful as an epsilon toxin prophylaxis or therapeutic. To date, the specific identity of the epsilon toxin receptor remains unknown. The receptor is perhaps a heat-labile sialoglycoprotein, because pretreatment of rat synaptosome membranes with heat (70–80°C for 10 minutes), neuraminidase, or pronase effectively reduced the binding of epsilon toxin.²²⁴ Furthermore, the same study revealed that a snake presynaptic neurotoxin (beta-bungarotoxin) decreases epsilon toxin binding in a dose-dependent fashion, suggesting a common (unidentified) receptor. In contrast, the presynaptic neurotoxin produced by *C botulinum* type A had no effect upon binding of the epsilon toxin. Knowledge of the receptor and how it interacts with the epsilon toxin would be useful in formulating effective, receptor-based therapies.

Although they are readily available and commonly used in the field,²²⁵ veterinary vaccines for *C perfrin-*

gens and associated toxins, like many other veterinary vaccines, are often formaldehyde toxoids consisting of various antigens from culture filtrates or even whole cell cultures. These vaccines are efficacious and cost-effective for animals but are generally considered too crude for human use. Any human epsilon toxin vaccine will likely be chemically (ie, formaldehyde) or recombinantly (ie, mutation of critical residues needed for receptor binding or heptamerization) detoxified versions of purified protein. The latter concept of recombinantly attenuating a toxin to generate a vaccine has been used successfully for other bacterial toxins, including the *S aureus* enterotoxins²²⁶ such as staphylococcal enterotoxin B, which is on the category B list of select agents. The technique used by Ulrich et al²²⁶ for generating recombinant vaccines against *S aureus* enterotoxins involved data from X-ray crystal structures of the toxin and major histocompatibility complex class II receptors, molecular modeling of toxin binding to the receptor, and the recombinant alteration of the specific toxin residues important for receptor interactions. This approach may prove useful for generating efficacious epsilon toxin vaccines pending the difficult process of receptor identification and crystallization.

In 1992 Hunter et al accomplished the cloning, sequencing, and expression of the gene, an important step toward a purified vaccine suitable for use in humans.²²⁷ Earlier studies by Sakurai et al,²²⁸ which showed through chemical modification that certain amino acids are essential for lethality, set the stage for subsequent alteration of select residues through recombinant technology. Oyston et al have taken another major step toward a recombinant vaccine for epsilon toxin by substituting a proline for the histidine at residue 106 of the toxin.²²⁹ This recombinant molecule is nontoxic in vitro as well as in vivo, and affords protection as a vaccine in mice against a 100 LD₅₀ of toxin given intravenously. X-ray crystallography of a toxin-receptor complex would also likely yield definitive, useful data for a better recombinant vaccine. Furthermore, it is evident that a single epitope on epsilon toxin can elicit protection against the toxin or the bacterium, as shown by immunization of mice or rabbits with a monoclonal antibody that generates antiidiotypic antibodies.²³⁰ Clearly, a refined vaccine should ultimately provide a useful prophylaxis for humans against *C perfringens* epsilon toxin. With renewed interest in and funding opportunities for select agents such as *C perfringens* epsilon toxin, various researchers from around the world should quickly solve the protein's mysteries and generate more efficacious therapies as well as vaccines suitable for human use.

SUMMARY

Exposure to harmful biological 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 small amounts of toxins typically produced by organisms. Biological toxins may be more suitable for causing incapacitation or death among small groups or for assassinations. The biological toxins presented in this chapter are diverse in structure and mode of action. Proper diagnosis and care represent a daunting challenge for physicians.

Trichothecene mycotoxins are toxic to humans and a host of other organisms by inhibiting DNA, RNA, and protein synthesis. Local route-specific effects include necrosis and inflammation. Systemic toxic responses are similar, regardless of the exposure route. Treatment relies on decontamination and symptom-based supportive care. There have been unconfirmed reports of trichothecene mycotoxins used as weapons in Southeast Asia.

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.

Epsilon toxin of *C perfringens*, a protein responsible for animal enterotoxemias, is rapidly fatal in various animal models. The toxin causes pore formation in cell membranes, ion imbalance, and cytoskeletal disruption, leading to cell death. Although it has not been implicated in human disease, epsilon toxin causes severe symptoms in animals including diarrhea, respiratory distress, and convulsions. A vaccine exists for veterinary use, but there is no specific therapy for epsilon intoxication.

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Chapter 18

LABORATORY IDENTIFICATION OF BIOLOGICAL THREATS

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SUMMARY

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INTRODUCTION

The ability of military laboratories to identify and confirm the presence of biological threats has significantly improved over the past decade. Identification approaches have advanced from classical identification methods performed in only a few reference laboratories to complex integrated diagnostic systems that are maturing as part of the Joint Biological Agent Identification and Diagnostic System (JBAIDS) for field laboratories. During the Persian Gulf War (1990–1991), deployed field laboratories and environmental surveillance units depended significantly on immunoassay methods with limited sensitivity and specificity. Because of intensive efforts by scientists at military reference centers, such as the US Army Medical Research Institute of Infectious Diseases (USAMRIID), the Naval Medical Research Center, the Armed Forces Institute of Pathology, and the US Air Force Institute for Operational Health, researchers are better prepared to identify and confirm the presence of the highest priority biological threats to human health (Exhibit 18-1).^{1,2} However, the biological

threat is more complicated than ever before. Future diagnostic and identification systems will depend on an integrated set of technologies, including new immunodiagnostic assays and rapid gene analysis methods to detect a broad spectrum of possible biological markers for diagnosing biological threats (see Exhibit 18-1).² The combination of several diagnostic approaches will improve reliability and confidence in laboratory results, which may shape medical treatment or response after an attack. Military and civilian clinical laboratories are now linked into a laboratory response network (LRN) for bioterrorism sponsored by the Centers for Disease Control and Prevention (CDC).³ Together, these efforts have improved the national preparedness, 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 the agent identification approaches and state-of-the-art diagnostic technologies available to protect and sustain the health of soldiers and other military personnel.

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. For the purposes of this chapter, a biological threat is any infectious disease entity or biological toxin intentionally delivered by opposing forces to deter, delay, or defeat US or allied military forces in the accomplishment of the mission. 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 and field laboratories is to confirm the clinical diagnosis of the medical officer, laboratory staff also provide subject matter expertise in theaters of operation on the handling and identification of hazardous microorganisms and biological toxins. Because these laboratories have a global view of disease in the theater, 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 a comprehensive environmental or preventive medicine surveillance system in a theater of operations.

Military Field Laboratories

If a complete medical treatment facility 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 which the health service element is task organized for a specific mission. In this case, medical laboratory support should be provided by a facility outside the area of operations.⁴ Army medical treatment facilities in a theater of operations have limited microbiology capabilities unless supplemented with a microbiology augmentation set (M403), which is fielded with an infectious disease physician, a clinical microbiologist, and a laboratory technician. The M403 set contains all of the necessary equipment and reagents to identify commonly encountered pathogenic bacteria and parasites, evaluate bacterial isolates for antibiotic sensitivity, and screen for some viral infections. Although this medical set does not contain an authoritative capability for definitively identifying biological warfare agents, it supports ruling out common infections. Specimens requiring more comprehensive analysis capabilities are forwarded to the nearest reference or confirmatory laboratory. After the Persian Gulf War, all of the military services recognized a need to develop additional deployable laboratory assets to support biological threat identification and preventive medicine efforts (described below).

Army

Army medical laboratories (AMLs) are modular, task-organized, and corps-level assets providing

EXHIBIT 18-1**REGULATED BIOLOGICAL SELECT AGENTS AND TOXINS****US DEPARTMENT OF HEALTH AND HUMAN SERVICES SELECT AGENTS AND TOXINS**

Abrin
 Cercopithecine herpesvirus 1 (Herpes B virus)
Coccidioides posadasii
 Conotoxins
 Crimean-Congo hemorrhagic fever virus
 Diacetoxyscirpenol
 Ebola virus
 Lassa fever virus
 Marburg virus
 Monkeypox virus
 Reconstructed replication competent forms of the 1918 pandemic influenza virus containing any portion of the coding regions of all eight gene segments (Reconstructed 1918 Influenza virus)
 Ricin
Rickettsia prowazekii
Rickettsia rickettsii
 Saxitoxin
 Shiga-like ribosome inactivating proteins
 South American Haemorrhagic Fever viruses
 Flexal
 Guanarito
 Junin
 Machupo
 Sabia
 Tetrodotoxin
 Tick-borne encephalitis complex (flavi) viruses
 Central European Tick-borne encephalitis
 Far Eastern Tick-borne encephalitis
 Kyasanur forest disease
 Omsk hemorrhagic fever
 Russian Spring and Summer encephalitis
 Variola major virus (Smallpox virus) and Variola minor virus (Alastrim)
Yersinia pestis

OVERLAP SELECT AGENTS AND TOXINS

Bacillus anthracis
 Botulinum neurotoxins
 Botulinum neurotoxin producing species of *Clostridium*
Brucella abortus
Brucella melitensis
Brucella suis
Burkholderia mallei (formerly *Pseudomonas mallei*)
Burkholderia pseudomallei (formerly *Pseudomonas pseudomallei*)
Clostridium perfringens epsilon toxin
Coccidioides immitis
Coxiella burnetii

Eastern equine encephalitis virus
Francisella tularensis
 Hendra virus
 Nipah virus
 Rift Valley fever virus
 Shigatoxin
 Staphylococcal enterotoxins
 T-2 toxin
 Venezuelan equine encephalitis virus

US DEPARTMENT OF AGRICULTURE SELECT AGENTS AND TOXINS

African horse sickness virus
 African swine fever virus
 Akabane virus
 Avian influenza virus (highly pathogenic)
 Bluetongue virus (Exotic)
 Bovine spongiform encephalopathy agent
 Camel pox virus
 Classical swine fever virus
Cowdria ruminantium (Heartwater)
 Foot-and-mouth disease virus
 Goat pox virus
 Japanese encephalitis virus
 Lumpy skin disease virus
 Malignant catarrhal fever virus (Alcelaphine herpesvirus type 1)
 Menangle virus
Mycoplasma capricolum / M.F38 / *M. mycoides Capri* (contagious caprine pleuropneumonia)
Mycoplasma mycoides mycoides (contagious bovine pleuropneumonia)
 Newcastle disease virus (velogenic)
 Peste des petits ruminants virus
 Rinderpest virus
 Sheep pox virus
 Swine vesicular disease virus
 Vesicular stomatitis virus (Exotic)

US DEPARTMENT OF AGRICULTURE PLANT PROTECTION AND QUARANTINE (PPQ) SELECT AGENTS AND TOXINS

Candidatus Liberobacter africanus
Candidatus Liberobacter asiaticus
Peronosclerospora philippinensis
Ralstonia solanacearum race 3, biovar 2
Schlerophthora rayssiae var *zeae*
Synchytrium endobioticum
Xanthomonas oryzae pv. *oryzicola*
Xylella fastidiosa (citrus variegated chlorosis strain)

Reproduced from: US Department of Health and Human Services and US Department of Agriculture Select Agents and Toxins, 7 CFR Part 331, 9 CFR Part 121, and 42 CFR Part 73. Available at: <http://www.cdc.gov/od/sap/docs/salist.pdf>. Accessed February 23, 2006.

comprehensive preventive medicine laboratory support to theater commanders. AMLs are capable of testing environmental and clinical specimens for a broad range of biological, chemical, and radiological hazards. For biological agents, the laboratory uses a variety of rapid analytical methods, such as real-time PCR, electrochemiluminescence (ECL), enzyme-linked immunosorbent assay (ELISA), and more definitive analyses involving bacterial culture, fatty acid profiling, and necropsy and immunohistochemistry.² AMLs have significant “reach back” capability to reference laboratories in the continental United States (CONUS) for support. The largest of the service laboratories, AMLs can identify “typical” infectious diseases including endemic disease threats and they contain redundant equipment for long-term or split-base operations. The laboratory contains all of the necessary vehicles and equipment to move and maintain itself in the field.

Navy

The Navy’s forward deployable preventive medicine units (FDPMUs) are medium-sized mobile laboratories using multiple rapid techniques (polymerase chain reaction [PCR] and ELISA) for identifying biological warfare agents on the battlefield. The FDPMUs are also modular and have the ability to analyze samples containing chemical and radiological hazards. These laboratories specialize in identifying biological threat agents in concentrated environmental samples (high confidence), but they can also identify endemic infectious disease in clinically relevant samples.

Air Force

Air Force biological augmentation teams (AFBATs) use rapid analytical methods (such as real-time PCR) to screen environmental and clinical samples for threat agents. The teams are small (two persons), easily deployed, and designed to fall in on preexisting or planned facilities. The units are capable of providing early warning to commanders of the potential presence of biological threat agents.

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. A critical but little understood concept is that the rapid recognition of biological warfare threats must be fully integrated with preventive medicine activities and the response to endemic infectious diseases.

Laboratory Response Network

The response to future biological threats will require the entire military laboratory network. 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 “listen to the hoof beats” of medical diagnosis, rule out the most common of threats, and alert the public health network about suspicious disease occurrences. The military LRN consists of the front-line medical treatment facility clinical laboratories or deployed AMLs backed by regional medical treatment facilities 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 pyramid are research laboratories, such as USAMRIID (Fort Detrick, Md) and the Naval Medical Research Center (Silver Spring, Md). Other laboratories, such as the Armed Forces Institute of Pathology (Washington, DC) and the US Air Force Institute for Operational Health (San Antonio, Texas) also provide reference laboratory services for endemic infectious diseases. Military research laboratories are best used to solve the most complex and difficult diagnostic problems, because usually they are not organized to perform high-throughput clinical sample processing and evaluation. Sentinel laboratories are generally supported by the network’s designated confirmatory laboratories but may communicate directly with national laboratories when hemorrhagic fevers or orthopoxviruses (ie, smallpox virus) are suspected. 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 18-1).

Biosafety and Biosecurity in the Military Clinical and Field Medical Laboratories

Biosafety Considerations

Specific guidelines for handling hazardous agents are contained in “Biosafety in Microbiological and Biomedical Laboratories” published by the US Department of Health and Human Services (DHHS).⁵ By avoiding the creation of aerosols and using certain safety practices, most bacterial threats can be handled using standard microbiological practices at biosafety level (BSL) 2. BSL-2 conditions require that laboratory

personnel have specific training in handling pathogenic agents and are directed by competent scientists. Access to BSL-2 laboratories is restricted when work is being conducted and safety precautions are taken with contaminated sharp items. Procedures that may create infectious aerosols are conducted only in biological safety cabinets or other physical containment equipment. When samples must be processed on a bench top, laboratory personnel must use other primary barrier equipment, such as plexiglass shields, protective eyewear, lab coat and gloves, and work in low-traffic areas with minimum air movement. BSL-3 conditions, which consist of additional environmental controls (ie, negative pressure laboratories) and procedures, are intended for work involving indigenous or exotic agents that may cause serious or potentially lethal disease from inhalational exposure. Limited prophylactic vaccines and therapeutics may be available to treat exposed personnel in case of an accident. BSL-4 conditions are reserved for the most dangerous biological agents for which specific medical interventions are not available and an extreme risk for aerosol exposure exists. BSL-4 requires the use of negative pressure laboratories and one-piece, positive-pressure personnel suits ventilated by a life support system. Laboratory personnel should incorporate universal bloodborne pathogen precautions and follow the guidelines outlined in federal regulation 29 Code of Federal Regulations (CFR) 1910.1030, "Occupational Exposure to Blood-borne Pathogens."⁶ Specific precautions for each of the highest priority biological threats can be found in the Basic Protocols for Level A (Sentinel) Laboratories (<http://www.bt.cdc.gov> or <http://www.asm.org>).

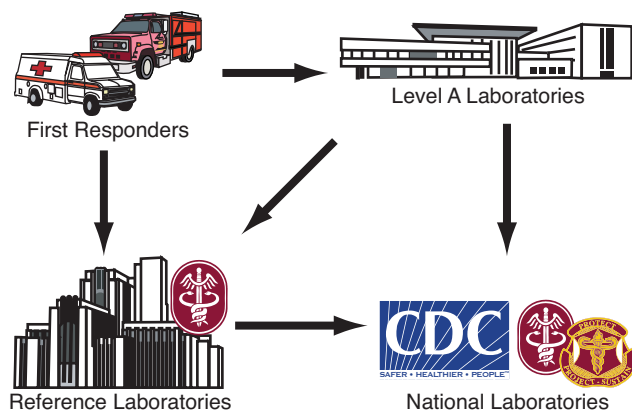


Fig. 18-1. The network of military laboratories with connections to federal and state civilian response systems provides unparalleled depth and resources to the biological threat response.

Biosurety

The 2001 anthrax letter attacks, which resulted in 22 cases of cutaneous or inhalational anthrax and five deaths, raised the national concern about the safety and security of laboratory stocks of biological threats in government, commercial, and academic laboratories.⁷ As a result, the DHHS promulgated new regulations (42 CFR, Part 73) that provided substantial controls over access to biological select agents and toxins (BSATs), required registration of facilities, and established processes for screening and registering laboratory personnel.⁸ DHHS and the US Department of Agriculture (USDA) identified over 80 biological agents that required these regulatory controls (see Exhibit 18-1). In addition to federal regulations, the US Department of Defense (DoD) directed additional controls for access to BSATs and required the establishment of biosurety programs. These actions were taken to foster public trust and assurance that BSATs are handled safely and securely in military laboratories. Among the services, the Army has established the most comprehensive set of draft regulations (AR 50-XX) with implementing memoranda.

At USAMRIID the framework for the military biosurety program was derived from the DoD's experience with chemical and nuclear surety programs.⁹⁻¹¹ These surety programs incorporate reliability, safety, and security controls to protect particular chemical and nuclear weapons. The DoD biological surety program applies many of the same controls as the chemical and nuclear surety programs to medical biological defense research and exceeds the standards of biosecurity programs in other federal and nonfederal laboratories.

Every military facility that stores and uses BSATs must be registered not only with the CDC (see 42 CFR Part 73) but also with the DoD.^{8,9} In the case of Army laboratories, registrations are completed through the Assistant Secretary of the Army (Installation and Environment). Army clinical laboratories, especially those participating in the LRN triservice initiative, are coordinated through the Army Medical Command health policy and services. Not all clinical laboratories need to be registered. However, unregistered laboratories must follow the 42 CFR 73 "Clinical Laboratories Exemption," which states that clinical laboratories identifying select agents have 7 days to forward or destroy them. The transfer of BSAT cultures requires the exchange of transfer documents (ie, CDC/APHIS Form 2) between CDC-registered facilities.

Laboratory directors who supervise activities that stock BSATs must be prepared to implement a variety of stringent personnel, physical security, safety, and agent-inventory guidelines. The law established penalties of

TABLE 18-1
KEY IDENTITY MARKERS FOR SELECTED BIOLOGICAL SELECT AGENTS AND TOXINS

Biological Select Agent and Toxin	Key Identity Markers	Biosafety Level*	Confirmatory Methods
Anthrax	Gram-positive rod; spore-forming; aerobic; nonmotile; catalase positive; large, gray-white to white; nonhemolytic colonies on sheep blood agar plates	2	<ul style="list-style-type: none"> • Gamma phage sensitivity • Immunohistochemistry • PCR
Botulism	Gram-positive rod; spore-forming; obligate anaerobe catalase negative; lipase production on egg yolk agar; 150,000 dal protein toxin (types A,B,C,D,E,F,G); 2 subunits	2	<ul style="list-style-type: none"> • Immunoassay • Mouse neutralization assay • PCR
Plague	Gram-negative coccobacilli often pleomorphic; nonspore forming; facultative anaerobe; nonmotile beaten copper colonies (MacConkey's agar)	2	<ul style="list-style-type: none"> • Immunofluorescence assay • PCR
Smallpox	Large double-stranded DNA virus; enveloped, brick-shaped morphology; Guarnieri bodies (virus inclusions) under light microscopy	4	<ul style="list-style-type: none"> • PCR • EM • Immunohistochemistry • Immunoassay
Tularemia	Extremely small, pleomorphic, gram-negative coccobacilli; nonspore forming; facultative intracellular parasite; nonmotile; catalase positive opalescent smooth colonies on cysteine heart agar	2	<ul style="list-style-type: none"> • PCR • Immunoassay
Ebola	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	4	<ul style="list-style-type: none"> • PCR • EM • Immunoassay • Immunohistochemistry
Marburg	Morphologically identical to Ebola virus	4	<ul style="list-style-type: none"> • PCR • EM • Immunoassay • Immunohistochemistry
Viral encephalitides	Linear positive-sense single-stranded RNA virus; enveloped, spherical virions with distinct glycoprotein spikes; cytopathic effect in Vero cells	3	<ul style="list-style-type: none"> • PCR • EM • Immunoassay • Immunohistochemistry
Ricin toxin	60,000–65,000 dal protein toxin; 2 subunits castor bean origin	2	<ul style="list-style-type: none"> • Immunoassay

Data sources: (1) Burnett JC, Henchal EA, Schmaljohn AL, Bavari S. The evolving field of biodefense: therapeutic developments and diagnostics. *Nat Rev Drug Discov.* 2005;4:281–297. (2) Henchal EA, Teska JD, Ludwig GV, Shoemaker DR, Ezzell JW. Current laboratory methods for biological threat agent identification. *Clin Lab Med.* 2001;21:661–678.

*BSL-2 bacterial agents must be handled at BSL-3 with additional precautions or in a biological safety cabinet if laboratory procedures might generate aerosols.

EM: electron microscopy

PCR: polymerase chain reaction

up to \$250,000 (individual) or \$500,000 (organization) for each violation. Enhanced safety procedures are required to work with or store BSATs. The DoD Biological Defense Safety Program is codified in Title 32 United States Code Part 627 and published as Army

Regulation 385-69. Guidelines for the safe handling of BSATs can be found in CDC guidelines “Biosafety in Microbiological and Biomedical Laboratories.”¹⁵ Although many bacterial agents can be handled in the BSL-2 clinical laboratory (Table 18-1), most work

requires at least a class II biological safety cabinet or hood and BSL-3 practices if there is a potential to create aerosols.⁵ Biosurety guidelines require that personnel complete biological safety training before having access to BSATs. A key goal of the guidelines is to prevent access to BSATs by unauthorized personnel. In addition to locked doors and freezers, continuous monitoring of areas where BSATs are held is required. Moreover, the capability to respond to the loss of agent must be incorporated into a response plan. Physical security of a facility by armed guards who can respond in minutes is a component of Army regulations.

Perhaps the most controversial of the DoD and Army guidelines is the requirement for a personnel reliability program, which requires that reviewing officials (usually the military unit commander, laboratory director, or otherwise delegated officer) aided by certifying officials (or employee supervisors) review the suitability of every staff member with access to BSATs with regard to behavioral tendencies, characteristics,

medical history, financial history, work habits, attitude, training, and more. Additionally, employees are actively screened for illegal drug use through urinalysis and alcohol abuse by observation. The biosurety personnel reliability program incorporates the requirements of the chemical and nuclear surety programs, which were not incorporated into federal law (except for the need for national agency and credit checks). The DoD views the personnel reliability program as essential because threat assessments have identified the lone disgruntled insider as the most serious threat to the biodefense program. On-site and off-site contractors who support DoD programs must implement the same safeguards under the current policies. These regulations may seem excessive because many BSATs can be obtained from natural sources; however, the DoD and the Army provided these guidelines to minimize risks associated with the release of a high-consequence pathogen from military facilities.

IDENTIFICATION APPROACHES

Specimen Collection and Processing

Clinical specimens can be divided into three different categories based on the suspected disease course: (1) early postexposure, (2) clinical, and (3) convalescent.¹² The most common specimens collected include nasal and throat swabs, induced respiratory secretions, blood cultures, serum, sputum, urine, stool, skin scrapings, lesion aspirates, and biopsy materials.² Nasal swab samples should not be used for making decisions about individual medical care; however, they should support the rapid identification of a biological threat (post-attack) and subsequent epidemiological surveys.^{13,14} After overt attacks with a suspected biological agent, baseline serum samples should be collected on all exposed personnel. In the case of suspicious deaths, pathology samples should be taken at autopsy to assist in outbreak investigations. Specimens and cultures containing possible select biological agents should be handled in accordance with established biosafety precautions. Specimens should be sent rapidly (within 24 hours) to the analytical laboratory on wet ice at 2°C to 8°C. Blood cultures should be collected before the administration of antibiotics and shipped to the laboratory within 24 hours at room temperature (21°C–23°C). Blood culture bottles incubated in continuous monitoring instrumentation should be received and placed within 8 hours of collection. Overseas (OCONUS) laboratories should not attempt to ship clinical specimens to CONUS reference laboratories using only wet ice. Shipments requiring more than 24 hours should be

frozen on dry ice or liquid nitrogen. Specific shipping guidance should be obtained from the supporting laboratory before shipment. Specimens for complex analysis, such as gene amplification methods, should not be treated with permanent fixatives (eg, formalin or formaldehyde). International, US, and commercial regulations mandate the proper packing, documentation, and safe shipment of dangerous goods to protect the public, airline workers, couriers, and other persons who work for commercial shippers and who handle the dangerous goods within the many segments of 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. Specific specimen collection and handling guidelines for the highest priority bioterrorism agents are available from CDC and the American Society for Microbiology (see <http://www.bt.cdc.gov> or <http://www.asm.org>).

Clinical Microbiological Methods

Laboratory methods for biological threat agent identification were previously reviewed in this chapter.^{2,15} Specific LRN guidelines for identifying the highest priority (category A) bioterrorism agents can be obtained from the CDC (<http://www.bt.cdc.gov>). The physician's clinical observations and direct smears of clinical specimens should guide the analytical plan (see Table 18-1).^{2,15} Most aerobic bacterial threat agents can

be isolated by using four bacteriological media: (1) 5% sheep blood agar (SBA), (2) MacConkey agar (MAC), (3) chocolate agar (CHOC), and (4) cystine heart agar (CHA) supplemented with 5% sheep blood. Nonselective SBA supports the growth of *Bacillus anthracis*, *Brucella*, *Burkholderia*, and *Yersinia pestis*. MAC agar, which is the preferred selective medium for gram-negative *Enterobacteriaceae*, supports *Burkholderia* and *Y. pestis*. CHA is the preferred medium for *Francisella tularensis*, but CHOC agar also suffices. A liquid medium, such as thioglycollate broth or trypticase soy broth, can also be used followed by subculturing to SBA or CHOC when solid medium initially fails to produce growth. The selection of culture medium can be modified when the target microorganism is known or highly suspected; however, in most cases, the use of multiple media options is recommended. Liquid samples can be directly inoculated onto solid agar and streaked to obtain isolated colonies. Specific culture details for the highest priority biological threats are available from the CDC (www.bt.cdc.gov).

Antibiotic Susceptibility Testing

Screening for unique antibiotic resistance or susceptibility may be critical to recognizing organisms that acquire natural or directed enhancements. Multiple drug-resistant *Y. pestis*, *Brucella abortus*, and *Burkholderia* strains have been identified.¹⁶⁻²⁰ In addition to classical Kirby-Bauer disk diffusion antibiotic susceptibility tests or minimum inhibitory concentration determinations, a variety of commercial antibiotic susceptibility testing devices for use by community hospitals have been standardized to reduce the time required to achieve results.²¹⁻²⁴ Unfortunately, these more rapid tests may not always be optimum for detecting emerging resistance. Although standardization of protocols by the Clinical and Laboratory Standards Institute has ensured reproducibility of results, emerging technology for detecting resistance markers is not available in most clinical laboratories. In addition, detecting progressive stepwise resistance is limited to known and standardized techniques.²⁵ Molecular methods that could enhance screening for unique genetic markers of resistance have been developed²⁶⁻³⁰; however, genetic analysis approaches can be cumbersome when multiple loci are involved, as in the case of resistance to antibiotics related to tetracycline or penicillin.^{29,30} DNA microarrays offer the potential for simultaneous testing for specific antibiotic resistance genes, loci, and markers.^{28,29} Grimm et al differentiated 102 of 106 different TEM beta-lactamase variant sequences by using DNA microarray analysis.²⁹ However, a comprehensive database of

resistance genetic determinants for many biological threats is not available, and new loci may emerge. In response to the problem of emerging enteric diseases, an electronic network has been established to detect outbreaks of selected foodborne illnesses by using pulsed-field gel electrophoresis.^{31,32} Fontana et al demonstrated pulsed-field gel electrophoresis combined with ribotyping (a molecular method based on the analysis of restriction fragment length polymorphisms of ribosomal RNA genes) as an effective approach for detecting multidrug-resistant *Salmonella*.³² Applying these methods to the broader array of potential threats should be an intensive future research effort.

Immunodiagnostic Methods

An integrated approach to agent detection and identification, which is essential for a complete and accurate disease diagnosis, provides the most reliable laboratory data.² Immunodiagnostic techniques may play a key role in diagnosing disease by detection of agent-specific antigens and/or antibodies present in clinical samples. The most significant problem associated with the development of an integrated diagnostic system has been the inability of such 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, which could complicate medical decisions. However, recent 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.

Detecting specific protein 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 diagnosing the diseases they cause. Nearly all methods for detecting antigens and antibodies rely on the production of complexes made of one or more receptor molecules and the entity being detected.

Traditionally, assays for detecting proteins and other non-nucleic 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 other nonantibody molecules, including aptamers, peptides, and engineered antibody fragments, are now being used in affinity-based detection technologies.³³⁻⁴²

Diagnosing disease by immunodiagnostic technologies is a multistep process involving formation of complexes bound to a solid substrate. This process is like making a sandwich: detecting the biological agent or antibody depends on incorporating all the “sandwich” components. Elimination of any one part of the sandwich results in a negative response (Figure 18-2). The primary ligands used in most immunoassays are polyclonal or monoclonal antibodies or antibody fragments.

Binding one or more of the antibodies onto a solid substrate is usually the first event of the assay reaction cascade. Immunoassays can generally be termed as 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. However, 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.

The final step in any immunoassay is the 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, the reactants may be detected visually, electronically, chemically, or physically. Because a single enzyme molecule can catalyze many chemical reactions without being consumed in the reaction, these labels are effective at amplifying assay signals. Most common enzyme-substrate reactions used in immunodiagnosics 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 from the intrinsic fluorescent

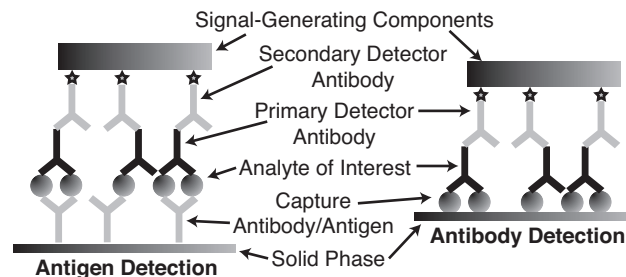


Fig. 18-2. Standard Sandwich Immunoassay. Detecting the biological agent or antibody depends on incorporating all the “sandwich” components. Elimination of any one part of the sandwich results in a negative response.

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 of these labels, which can be visualized by the naked eye or by instruments, are stable under a variety of environmental conditions. However, because these labels are essentially inert, they do not produce an amplified signal. Signal amplification is useful and desirable because it results in increased assay sensitivity.

Advances in 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. The following overview discusses technologies currently used for identifying biological agents and also used (or under development) for diagnosing the diseases caused by these agents.

Enzyme-Linked Immunosorbent Assay

Since the 1970s the ELISA has remained a core technology for diagnosing disease caused by a wide variety of infectious and noninfectious agents. As a result, the ELISA is perhaps the most widely used and best understood immunoassay technology. Developed in many formats, assays can be designed to detect either antibodies produced in response to infection or antigens associated with the agents themselves. ELISAs that detect biological agents or agent-specific antibodies are heterogeneous assays in which an agent-specific antigen or host-derived antibody is captured onto a plastic multi-well plate by an antibody or antigen previously bound to the plate surface (capture moiety). Bound antigen or antibody is then detected using a secondary antibody (the detector antibody). The detector antibody can be directly labeled with a

signal-generating molecule or it can be detected with another antibody labeled with an enzyme. These enzymes catalyze a chemical reaction with substrate, which results in a colorimetric change. The intensity of this color can be measured by a modified spectrophotometer that determines the optical density of the reaction by 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 advantage of ELISAs is their ability to be configured for a variety of uses and applications. Use of ELISAs in field laboratory settings is possible but does require certain fixed-site logistical needs, such as controlled temperature incubators and refrigerators, the power needed to run them, 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 quantification difficult, and are relatively slow.

The DoD has successfully developed antigen-detection ELISAs for nearly 40 different biological agents

and antibody-detection ELISAs for nearly 90 different agents. All of these assays were developed by using the same solid phase buffers and other reagents, incubation periods, incubation temperatures, and general procedures (Table 18-2). Although there is significant variation in assay limits of detection, ELISAs typically are capable of detecting as little as 1 ng of antigen per mL of sample.

Electrochemiluminescence

Among the most promising new immunodiagnostic technologies is a method based on electrochemiluminescence (ECL) detection. One ECL system makes use of antigen-capture assays and a chemiluminescent label (ruthenium [Ru]) and includes magnetic beads to concentrate target agents. These beads are coated with capture antibody, and in the presence of biological agent, immune complexes are formed between the agent and the labeled detector antibody. Because of its small size (1,057 kDa), Ru can be easily conjugated to any protein ligand by using standard chemistries without affecting immunoreactivity or solubility of the protein. The heart of the ECL analyzer is an electrochemical flow cell with a photodetector placed just above the electrode. A magnet positioned just below the electrode captures the magnetic-bead-Ru-tagged

TABLE 18-2
COMPARISON OF IMMUNODIAGNOSTIC METHODS

	Enzyme-Linked Immunosorbent Assay	Dissociation- enhanced lanthanide fluorescence immunoassay time-resolved fluorescence	Electrochemi- luminescence	Flow-Based	Hand-Held Assay
Assay Parameters					
Incubation time	3.5 h	2.2 h	15 min	30 min	15 min
Number of steps	5	4	1	1	1
Detection method	Colorimetric	Fluorescence	Chemiluminescence	Fluorescence	Visual
Multiplexing	No	Potential	No	Yes	Potential
Key Performance Parameters					
Intra-assay variation (%)	15–20	20–50	2–12	10–25	Undetermined
Limit of detection: <i>Yersinia pestis</i> F1 (colony-forming units)	250,000	250	500	62,500	125,000
Limit of detection: Staphylococcal enterotoxin B (ng)	0.63	0.04	0.05	3.13	6.25
Limit of detection: Venezuelan equine encephalitis virus (plaque- forming units)	1.25 x 10 ⁷	3.13 x 10 ⁶	1.0 x 10 ⁷	3.13 x 10 ⁸	6.25 x 10 ⁸

immune complex and holds it against the electrode. The 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. The magnetic beads provide a greater surface area than conventional surface-binding assays like the ELISA. The reaction does not suffer from the 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 with a linear dynamic range can span six orders of magnitude.⁴³⁻⁴⁴

A field-ready ECL system consists of an analyzer and a personal computer with software. ECL systems possess several advantages, including speed, sensitivity, accuracy, and precision over a wide dynamic range. In a typical agent-detection assay, sample is added to reagents consisting of capture antibody-coated paramagnetic beads and a Ru-conjugated detector antibody. Reagents can be lyophilized. 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 min per sample cleaning and reading time). The system uses 96-well plates and is therefore able to handle large sample throughput requirements.

The ECL system has been demonstrated to be effective for detecting staphylococcal enterotoxin B, ricin toxin, botulinum toxin, *F tularensis*, *Y pestis* F1 antigen, *B anthracis* protective antigen, and Venezuelan equine encephalitis virus.^{2,45,46} The ECL system, which has been demonstrated in field settings, is used as one part of an integrated diagnostic system in several deployable and deployed laboratories. Critical assay performance characteristics and detection limits from three typical ECL agent-detection assays are shown in Table 18-2.

Time-Resolved Fluorescence

Time-resolved fluorescence (TRF) is an immunodiagnostic technology with assays available for detecting agent-specific antibodies, microorganisms, drugs, and therapeutic agents.⁴⁷⁻⁴⁹ In practice, TRF-based assays are sandwich-type assays similar to those used for ELISA. The solid phase is a micro-well plate coated in some manner with specific capture antibody (similar to that used with colorimetric ELISA platforms). However, instead of being labeled with enzymes, detector antibodies are labeled with lanthanide chelates. The technology takes advantage of the differential fluorescence lifespan of lanthanide chelate labels

compared to background fluorescence. The labels have an intense, long-lived fluorescence signal and a large Stokes shift, which result in an assay with a very high signal-to-noise ratio and high sensitivity.⁵⁰ Unlike ECL, TRF produces detectable fluorescence through the excitation of the lanthanide chelate by a specific wavelength of light. Fluorescence is initiated in TRF with a pulse of excitation energy, repeatedly and reproducibly. In 1 second, the fluorescent material can be pulse-excited 1,000 times with an accumulation of the generated signal. One TRF format is dissociation-enhanced lanthanide fluorescence immunoassay (DELFLIA) in which dissociation of the complex-bound chelate caused by adding a low-pH enhancement solution forms long-lasting fluorescent micelles. Detection limits as low as 10^{-17} moles of europium per well with a dynamic range of at least four orders of magnitude have been demonstrated.

The strength of DELFLIA assays derives from their sensitivity, similarity to the commonly used ELISA techniques, and potential for multiplexing. Four different lanthanides are available (europium, samarium, terbium, and dysprosium), and each has its own unique narrow emission spectrum.⁵¹ Both immunoassays and nucleic acid detection assays are compatible with this platform. Like the ECL assays, DELFLIA assays require purified high-quality antibodies. Critical assay performance characteristics and assay limits of detection from three typical DELFLIA agent detection assays are shown in Table 18-2. Although a field-ready version of this instrument is not available, the system is common to clinical laboratories and is used by the CDC-sponsored LRN.

Flow Cytometry

Flow cytometry, the measurement of physical and chemical characteristics of small particles, has many current research and healthcare applications 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 involves 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. The light is scattered, showing distribution and intensity characteristic of the particular sample. A wavelength of the light is selected that causes the dye, bound to the particle of interest, to fluoresce, and a computer counts or analyzes the fluorescent sample as it passes through the laser beam.

Using the same excitation source, the fluorescence may be split into different color components so that several different fluorophores can be measured simultaneously and the signals interpreted by specialized software. A number of multiplexed flow cytometry assays have been demonstrated.⁵² Particles can also be sorted from the stream and diverted into separate containers by applying a charge to the particles of interest.

One commercially available platform is a rapid assay system that reportedly can perform up to 100 tests simultaneously on a single sample. This system incorporates three familiar technologies: (1) bioassays, (2) microspheres, and (3) fluorescence. The system consists of a flow cytometer with a specific digital signal processing board and control software. Assays occur in solution, thus allowing for rapid reaction kinetics and shorter incubation times. 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, ie, it detects antigen in a typical antigen-detection assay. The instrument uses two lasers: one for detecting the microsphere itself, and the other for the detector. Microspheres, which 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, a dynamic range of three to four orders of magnitude, and highly consistent and reproducible results.⁵³ 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 be automated, can use tubes as well as 96- and 384-well plates. Many multiplexed assay kits are commercially available from a number of manufacturers for various cytokines, phosphoproteins, and hormones.

Critical assay performance characteristics and limits of detection from three typical flow-based agent-detection assays are shown in Table 18-2. No field-ready versions of these instruments are available, however, limiting the practical use of this plat-

form in deployment situations, and no commercial or DoD sources for biothreat agent assays are available for this platform.

Lateral Flow Assays

Commercially produced lateral flow assays, which have been on the market for many years, are so simple to use and interpret that some types are approved for over-the-counter use by the US Food and Drug Administration. Lateral flow assays 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 a visible marker element is placed on a sample application pad. As the sample flows across the membrane, antigen or antibody present in the sample binds to the labeled antibody and is captured as the complex passes the bound antibody or antigen (Figure 18-3). 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.

One of the greatest advantages of lateral flow assays is their lack of reliance on instrumentation and the associated logistical needs. However, this lack of instrumentation decreases the utility of the tests because results cannot be quantified. To respond to this deficiency, several technologies are being developed to make these assays more quantitative (they also increase the assays' sensitivity). One technology allows for quantitative interpretation of the lateral flow assay.⁵⁴ Another method for quantitative detection of antibody/antigen complex formation in lateral flow assays uses up-converting phosphors.^{55,56} Paramagnetic particles have similarly been used in assays and instruments capable of detecting changes in magnetic flux within the capture zone, improving sensitivity by as much as several orders of magnitude over more traditional lateral flow assays.

Lateral flow assays are commonly used by the DoD for detecting biological threat agents. In addition, several 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 lateral flow assays is their inability to run a full spectrum of control assays on a single strip assay. Only flow controls are included with most lateral flow assays. These controls show that the conditions were correct for reagent flow across the membrane but do not indicate the ability of the assay to appropriately capture antigen.

Molecular Detection Methods

Polymerase Chain Reaction

Originally conceived in 1983 by Kary Mullis at the Cetus Corporation,⁵⁷ polymerase chain reaction (PCR) became a reality only 2 years later with the publication by Saiki et al of its first practical application.⁵⁸ This first description of PCR by Mullis et al marked a milestone in biotechnology and the beginning of the field now known as molecular diagnostics. PCR is a simple, in-vitro chemical reaction that permits the synthesis of almost limitless quantities of a targeted nucleic acid sequence. At its simplest, the PCR consists of target DNA (also called template DNA), two oligonucleotide primers that flank the target DNA sequence to be amplified, a heat-stable DNA polymerase, a defined solution of salts, and an equimolar mixture of deoxyribonucleotide triphosphates (dNTPs). The mixture is then subjected to repeated cycles of defined temperature changes that help to facilitate denaturation of the template DNA, annealing of the primers to the target DNA, and extension of the primers so that the target DNA sequence is replicated. A typical PCR protocol comprises 30 to 50 thermal cycles. Each time a cycle is completed, there is a theoretical doubling of the target sequence. Therefore, under ideal conditions, a single copy of a nucleic acid target can be multiplied over a billion-fold

after 30 cycles. 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. The PCR products are typically visualized as bands on an agarose gel after electrophoresis and staining with a DNA intercalating dye such as ethidium bromide or Sybr green.

In multiplex PCR, two or more sets of primers specific for different targets are included in the same reaction mixture, allowing for multiple target sequences to be amplified simultaneously.⁵⁹ The primers used in multiplexed reactions must be carefully designed to have similar annealing temperatures and lack complementarity. Multiplex PCR assays have played a larger role in human and cancer genetics than in the detection of infectious organisms, where they have proven more complicated to develop and often result in lower sensitivity than PCR assays using single primer sets.

Reverse Transcriptase-PCR

The PCR method described previously was designed to amplify DNA. However, many important human diseases are caused by viruses with an RNA genome. Therefore, reverse transcriptase PCR (RT-PCR) was developed to amplify specific RNA targets. In this process, extracted RNA is first converted to complementary

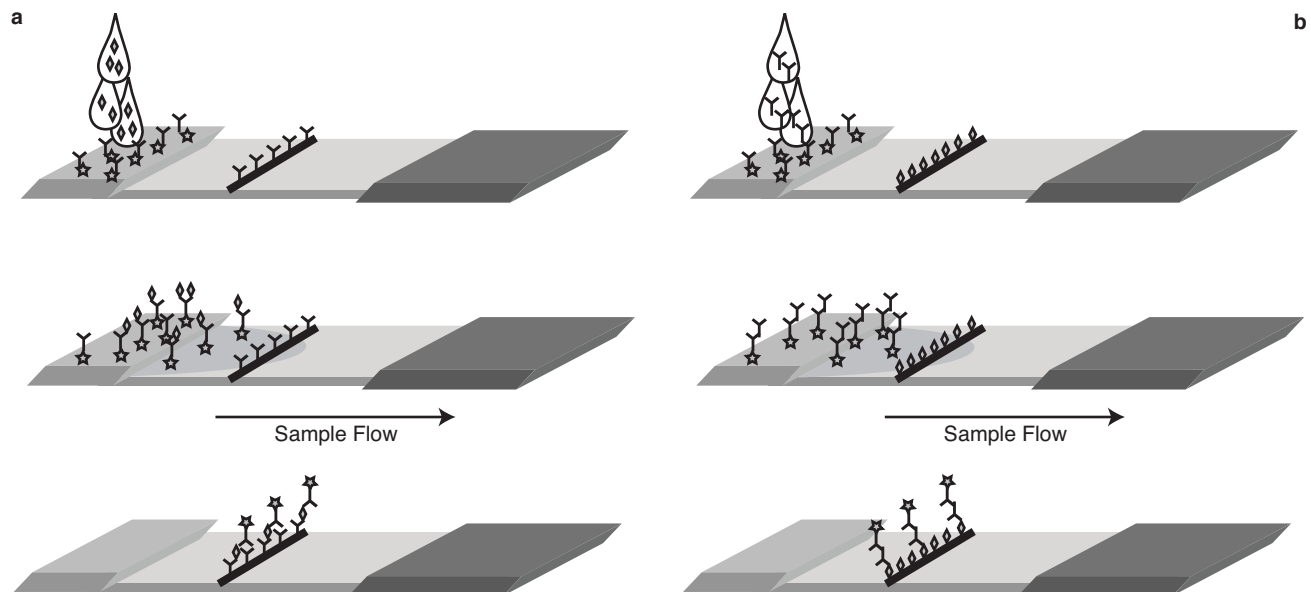


Fig. 18-3. Lateral flow assay format: A capture antibody (for antigen detection [a]) or antigen (for antibody detection [b]) is bound to the membrane, and a second antibody labeled with a visible marker element is placed on a sample application pad. As the sample flows across the membrane, antigen or antibody present in the sample binds to the labeled antibody and is captured as the complex passes the bound antibody or antigen.

DNA (cDNA) by reverse transcription, and then the cDNA is amplified by PCR. As originally described, reverse transcription of RNA into cDNA was carried out using retroviral RT enzymes from either avian myeloblastosis virus or Moloney murine leukemia virus. These enzymes are heat-labile and cannot be used at temperatures above about 42°C, which presents problems in terms of both nonspecific primer annealing and inefficient primer extension resulting from the potential formation of RNA secondary structures. These problems have largely been overcome by the development of a thermostable DNA polymerase derived from *Thermus thermophilus*, which, under the right conditions, can act as both a reverse transcriptase and a DNA polymerase.^{60,61} These and other similar enzymes can amplify RNA targets without the need for a separate RT step. Thus, this so-called “one-step” RT-PCR eliminates the need for the cumbersome, time consuming, and contamination-prone transfer of RT products to a separate PCR tube. Commercial RT-PCR assays are available for detecting a few important RNA viruses such as hepatitis C virus and human immunodeficiency virus, with numerous others published in the scientific literature as in-house or “home-brew” assays.

Real-Time PCR

By far the most important development in rapid identification of biological agents has been the development of “real-time” PCR methods. Although traditional PCR was a powerful analytical tool that launched a revolution in molecular biology, it was difficult to use in clinical and field laboratories. As originally conceived, gene amplification assays could take more than 5 to 6 hours to complete, not including the sample processing required before amplification. The improvement of assay throughput came with the development of assay chemistries that allowed the PCR reaction to be monitored during the exponential amplification phase on fast thermocyclers. Lee et al and Livak et al demonstrated assays based on the detection and quantification of fluorescent reporters that increased in direct proportion to the amount of PCR product in a reaction.^{62,63} By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during the exponential phase, in which the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. A significant increase in fluorescence above the baseline value measured during cycles 3 through 15 indicates the detection of accumulated PCR product. There are three main probe-based fluorescence-monitoring systems for DNA amplifica-

tion: (1) hydrolysis probes, (2) hybridization probes, and (3) DNA-binding agents. Hydrolysis probes most exemplified by TaqMan (Applied Biosystems, Foster City, Calif) chemistries have been the most successful for rapidly identifying biological threats. Probe hydrolysis assays use the fluorogenic 5' exonuclease activity of Taq polymerase.

Fast thermocycling was achieved first by using small volume assays in sealed capillary tubes placed in convection ovens and later by solid-state electronic modules.^{64,65} Optimal assay development coupled to instrument improvements has allowed the identification of selected biological agents within 20 to 40 minutes after specimen processing. Over 50 assays against 26 infectious agents have been developed using these approaches by the DoD, the CDC, and the US Department of Energy.² Commercially available rapid thermocycling instruments that can detect the fluorescent signals are now available from several sources, including Applied Biosystems (Foster City, Calif), Roche Diagnostics (Indianapolis, Ind), Idaho Technologies (Salt Lake City, Utah), Cepheid (Sunnyvale, Calif), and Bio-Rad (Hercules, Calif). The Idaho Technologies Ruggedized Advanced Pathogen Identification Device (RAPID) instrument has been incorporated into the first generation of the JBAIDS for use in field medical laboratories. By using new sample-processing techniques, the presumptive identification of most biological agents can be completed in 3 hours or less with rapid fluorescent-probe-based methods, compared to approximately 6 hours with older PCR methods. Other assay formats, such as fluorescent resonance energy transfer, have allowed the resolution of closely related species and mutation detection by characterizing the melting point of the detection probe.^{66,67} The demonstration of integrated sample preparation and gene amplification cartridges (such as Genexpert; Cepheid, Sunnyvale, Calif) has the potential to improve the reliability of PCR identification of biothreats by decreasing the need for extensive operator training and assay contamination.⁶⁸ Integrated cartridge gene amplification systems have been incorporated into the biohazard detection systems deployed to protect the US Postal Service.⁶⁹

TIGER

A significant obstacle for detecting future biothreats is the requirement of many technologies, such as immunoassays and most gene amplification methods, to have identified target biomarkers ahead of time. A unique coupling of broadly targeted gene amplification with mass-based detection of amplified products may allow for early recognition of replicating etiological agents without any preknowledge of

the targets. Sampath and Ecker have described the amplification of variable gene regions flanked by conserved sequences, followed by electrospray ionization mass spectrometry and base composition analysis of the products.^{70,71} This method, known as TIGER (triangulation identification for genetic evaluation of risks), provides for a high-throughput, multiple detection and identification system for nearly all

known, newly emergent, and bioengineered agents in a single test (<http://www.ibisrna.com/>; valid August 8, 2004). This rapid, robust, and culture-free system could have been used to identify agents such as SARS-related coronaviruses, before their recognition and characterization by traditional methods.⁷¹ Robust and portable TIGER systems are being developed for civilian and military applications.

EMERGING THREATS

The emergence of new biological threats is a particular challenge for the military clinical or field laboratory. For the past 50 years, the biological defense research program has focused on known or hypothesized collections of biological threats in the biological weapons program of the United States (ended in 1969) or of the former Soviet Union.^{72,73} However, several critical events have broadened the scope of the biological threat since 1984. First was the recognition after 1984 that nonstate actors might use biological agents in terrorist scenarios to advance political, religious, or social agendas (Table 18-3).⁷⁴⁻⁸⁰ These demonstrations suggest a more dangerous future because individuals or groups without any national allegiance use biological threats in small-scale scenarios outside of battlefield boundaries. Second, the discovery of an emerging biological weapons program in Iraq after the Persian Gulf War included several unexpected new threats, including aflatoxins, *Shigella*, and camelpox virus, in conjunction with historical biological threats, such as anthrax, ricin toxin, cholera, *Clostridium perfringens* and *C botulinum* neurotoxins.⁸¹ This discovery suggested that any etiological agent or combinations of biological agents, beyond those identified previously as optimal for past biological weapons of mass destruction, could be used by US adversaries to create fear and confusion. Third, the maturation and proliferation of biotechnology have resulted in several laboratory demonstrations of genetically engineered threats with new, potentially lethal characteristics.⁸¹⁻⁸⁵ Jackson et al demonstrated the virulence of orthopoxviruses enhanced by the insertion of immunoregulatory genes, such as interleukin-4.⁸² In other work, Athamna et al demonstrated the intentional selection of antibiotic-resistant *B anthracis*.⁸³ Borzenkov et al modified *Francisella*, *Brucella*, and *Yersinia* species by inserting beta-endorphin genes.^{84,85} 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.

TABLE 18-3
BIOTERRORISM INCIDENTS, 1984–2004

Biological Agent	Description
<i>Salmonella typhimurium</i>	Rajneeshee cult, The Dalles, Oregon, 1984 ¹
Ricin toxin	Patriots Council, Minnesota; Canada, 1991–1997 ^{2,3}
<i>Bacillus anthracis</i>	Aum Shinrikyo cult, Tokyo, Japan, 1995 ⁴
<i>Shigella dysenteriae</i>	Clinical lab, 1996 ⁵
Various	Hoax incidents, Nevada, 1997–1998 ⁶
<i>B anthracis</i>	Letters, Palm Beach, Florida; civilian news operations in New York City and in the Hart Senate Office Building, Washington, DC; also US postal facilities in the national capital area and in Trenton, NJ; 2001 ⁷
Ricin toxin	Manchester, England, 2002 ³ ; Possible Chechen separatist plan to attack the Russian embassy, London, England, 2003
Ricin toxin	Dirksen Senate Office Building, Mailroom serving Senate Majority Leader Bill Frist's office, Washington, DC, 2004 ³

Data sources: (1) Torok TJ, Tauxe RV, Wise RP, et al. A large community outbreak of salmonellosis caused by intentional contamination of restaurant salad bars. *JAMA*. 1997;278:389–395. (2) Mirarchi FL, Allswede M. CBRNE–ricin. *eMedicine* [serial online]. Available at: <http://www.emedicine.com/emerg/topic889.htm>. Accessed March 16, 2005. (3) Shea D, Gottron F. *Ricin: technical background and potential role in terrorism*. Washington, DC: Congressional Printing Office; February 4, 2004. Congressional Research Service Report RS21383. (4) Keim P, Smith KL, Keys C, Takahashi H, Kurata T, Kaufmann A. Molecular investigation of the Aum Shinrikyo anthrax release in Kameido, Japan. *J Clin Microbiol*. 2001;39:4566–4567. (5) Kolavic SA, Kimura A, Simons SL, Slutsker L, Barth S, Haley CE. An outbreak of *Shigella dysenteriae* type 2 among laboratory workers due to intentional food contamination. *JAMA*. 1997;278:396–398. (6) Tucker JB. Historical trends related to bioterrorism: an empirical analysis. *Emerg Infect Dis*. 1999;5:498–504. (7) Bush LM, Abrams BH, Beall A, Johnson CC. Index case of fatal inhalational anthrax due to bioterrorism in the United States. *N Engl J Med*. 2001;345:1607–1610.

These new threats will require the development of identification and diagnostic systems that can be flexibly used to allow early recognition of a unique

biothreat, representing one of the next major research and development challenges of the DoD and the National Institutes of Health.

BIOFORENSICS

Military clinical and field laboratories are not responsible for forensics protocols, which are required to support biocrime investigations and identify the origins of a biological threat. However, law enforcement personnel and military unit commanders may request the support of clinical laboratory experts and microbiologists to protect the nation's health and safety immediately after an attack. When allowed by command policy, military laboratories may assist in the evaluation of suspicious materials and rule out hoax materials if they use approved agent-identification protocols. Laboratories should not attempt to perform independent forensic analyses unless requested and supervised by appropriate law enforcement authorities. In CONUS, the intentional release of a biological threat is a crime and therefore is investigated by local and federal law enforcement agencies. OCONUS laboratories should coordinate closely with theater command staff and regional reference centers before conducting any analyses. At the national level, the US Department of Homeland Security National Bioforensic Analysis Center is responsible for providing highly regulated evaluations of biological threat materials from civilian and military sources. The Center also is responsible for establishing standards and coordinating analyses performed in supporting laboratories.

Although many clinical laboratories may be familiar with epidemiological investigations, bioforensic activities require a strict chain-of-custody and documentation process. Standards for analysis have been established by the American Society of Crime Laboratory Directors (see <http://www.fbi.gov/hq/lab/codis/forensic.htm>; accessed September 23, 2005). Related guidance can be found in International Organization for Standardization 17025 (Guide 25).⁸⁶ All laboratory activities must be directed to preserving the original evidence. Only validated analysis methods, in which the performance variables such as sensitivity, specificity, precision, robustness, and reliability have been scientifically peer reviewed, should be used. Laboratory protocols used in the CDC-sponsored LRN have been accepted by law enforcement officials for the analysis of evidentiary materials.

The biological and ecological complexities of most biothreat agents present forensic microbiologists with a number of significant analytical and interpretive challenges. Several available methods would be useful in

characterizing biocrime evidence. Classical phenotypic assays for physiological properties are among the most basic. Other methods include

- sequencing of DNA/RNA in samples and genomic sequencing of culture isolates;
- determination of phylogenetic patterns of single nucleotide polymorphisms from sequence data;
- association of microorganism genotypes with phenotypes;
- use of pathogenicity arrays (including 16S rRNA probes) to detect artificially constructed hybrid microorganisms; and
- use of screening tests for detection of antimicrobial resistance markers.

Use of multiple test methods is desirable to avoid misidentification of agents caused by induced or engineered mutations. To this end, portions of samples should be saved for additional investigation or confirmatory testing. Blind, barcoded sample replicates (eg, 10% of the replicates) are recommended.⁸⁷

Although the number of bioterrorism incidents has been small, integrated forensic and epidemiological approaches have assisted in past investigations. For example, a combination of epidemiological methods, classical phenotyping, and restriction endonuclease digest of marker plasmids contributed to the identification of a large community outbreak of salmonellosis caused by intentional contamination of restaurant salad bars.⁷⁴ The introduction of pulse field analysis of DNA from culture isolates helped to determine the magnitude and source of an outbreak of *Salmonella dysenteriae* type 2 among laboratory workers resulting from intentional food contamination.⁷⁶

Differentiation of *B anthracis* strains has been problematic because phenotypic and genetic markers are shared among the members of the *B cereus* family.⁸⁸ Worldwide clone-based diversity patterns have been demonstrated for *B anthracis*.⁸⁹ With the identification of variable number tandem repeats, identifying strains (unique genotypes) by multiple locus variable number tandem repeats analysis is now possible. Keim et al have suggested that there are about six major worldwide clonal lineages and nearly 100 unique types.^{89,90} Using these methods on *B anthracis*

spores that were aerosolized over Kameido, Japan, by the Aum Shinrikyo cult were identified as consistent with strain Sterne 34F2, which was used in Japan for protecting animals against anthrax.⁷⁹ Molecular subtyping of *B anthracis* played an important role in differentiating and identifying strains during the 2001 bioterrorism-associated outbreak.⁹¹ Because phylogenetic reconstruction using molecular data is often subject to inaccurate conclusions about phylogenetic relationships among operational taxonomic units, the analysis of single nucleotide polymorphisms, which exhibit extremely low mutation rates, may be more valuable for phylogenetic analyses. Using a remarkable set of 990 single nucleotide polymorphisms, Pearson et al demonstrated that nonhomoplastic, whole

genome single nucleotide polymorphism characters allowed branch points and clade membership for *B anthracis* laboratory reference strains to be estimated with great precision, providing greater insight into epidemiological, ecological, and forensic questions.⁹² These investigators determined the ancestral root of *B anthracis*, showing that it lies closer to a newly described “C” phylogenetic branch than to either of two previously described “A” or “B” branches. Similar analytical methods are evolving for characterizing strains of *Y pestis* and *F tularensis*.^{93,94} Continued maturation of genetic fingerprinting methods in the forensic environment can significantly deter biocrime and biological warfare in the future and result in more rapid identification of perpetrators.

FUTURE APPROACHES

Early Recognition of the Host Response

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. Studies suggest that 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.^{95,96} Poxviruses may possess several mechanisms to inhibit innate immunity.⁹⁷ Gibb et al 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.⁹⁸ Others have shown that Ebola virus infections are characterized by dysregulation of normal host immune responses.⁹⁹ 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.^{100,101} A complicated picture is emerging that goes beyond dysregulation of genes related to innate immunity. Relman et al suggested that there are genome-wide responses to pathogenic agents.¹⁰² Mendis et al identified cDNA fragments that were differentially expressed after 16 hours of in-vitro expo-

sure of human peripheral blood mononuclear cells to staphylococcal enterotoxin B.¹⁰³ By using custom cDNA microarrays and RT-PCR 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, and 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 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.

Joint Biological Agent Identification and Diagnostic System

An integrated diagnostic approach is required to recognize the biological threats of the future.² No single technology is sufficient to definitively identify any biological threat; thus, diagnostic systems must be able to detect multiple biological markers. Future systems must use a combination of immunological, gene amplification, and classical identification methods to identify important virulence factors, genus and species markers, common pathogenic markers, and antibiotic markers (Figure 18-4). The DoD is developing the JBAIDS as a flexible diagnostic platform that can incorporate a variety of new technologies.¹⁰⁴ JBAIDS will be a comprehensive integrated diagnostic

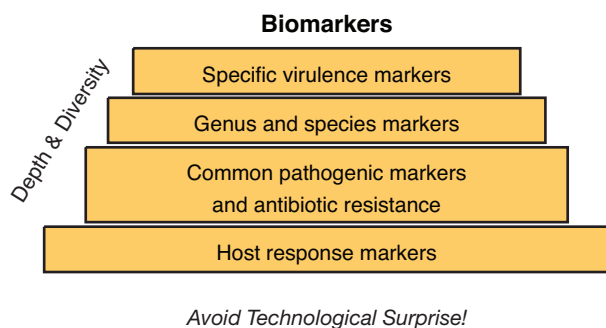


Fig. 18-4. Diagnostic systems must be able to detect multiple biological markers. No single technology is sufficient to definitively identify any biological threat. Future systems must use a combination of immunological, gene amplification, and classical identification methods to identify important virulence factors, genus and species markers, common pathogenic markers, and antibiotic markers.

platform capable of reliably identifying multiple biological threat agents and endemic infectious diseases. An acquisition strategy has been developed that will allow the integration of identification technologies into a single platform. Initial systems will include gene and antigen-detection systems linked to an interactive information-management framework. JBAIDS will support reliable, fast, and specific identification of biological agents from a variety of clinical and environmental sources and samples. JBAIDS will enhance healthcare by guiding the choice of appropriate treatments, effective preventive measures, and prophylaxis at the earliest stage of disease. In addition, JBAIDS will identify and quantify biological agents that could affect military readiness and effectiveness. Reliability, technological maturity, and supportability are the primary criteria used for selecting technologies included in JBAIDS.

SUMMARY

Protection of service members and their families from the effects of attack by biological agents requires the combined resources of the US military healthcare system and coordination with civilian public health officials. Military clinical and field laboratories serve as unique sentinels in CONUS and OCONUS areas for biological threats and emerging infectious diseases. Field laboratories in forward areas, which are equipped with the basic tools necessary to rule out endemic infectious diseases, can be augmented with the capability to identify the most likely biological warfare agents. CONUS military laboratories conform to standards and protocols established for the CDC-sponsored

LRN for the identification of biological threats. This response is supplemented by the comprehensive capabilities of the national laboratories, such as the CDC and USAMRIID, and military reference centers. Classical microbiology methods will remain as part of the core capability, which is being expanded to include integrated rapid immunodiagnostics and gene analysis technologies. The laboratory response for biological threats must be flexible to accommodate emerging and “nonclassical” agents. Future research will continue to develop real-time, simple, reliable, and robust methods that will be useable throughout the military healthcare and surveillance system.

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Chapter 19

CONSEQUENCE MANAGEMENT: THE NATIONAL AND LOCAL RESPONSE

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INTRODUCTION

Response to an intentional biological attack is likely to overwhelm local and regional healthcare facilities and resources, requiring the use of national assets to treat the infected and contain the disease. As stated in the biological incident annex of the National Response Plan (NRP), “No single entity possesses the authority, expertise, and resources to act unilaterally on the many complex issues that may arise in response to a disease outbreak and loss of containment affecting a multi-jurisdictional area.”¹ There must be coordination among healthcare facilities, local authorities, public health officials, state agencies, and federal agencies for an effective and efficient response to terrorism events. Biological response plans must be integrated at all levels, and cooperative efforts to leverage assets from nonaffected areas must be planned and exercised before the event. Critical tasks for healthcare facilities responding to an outbreak include treating the ill and preventing nosocomial spread of disease; however, facilities must also be prepared to expand surge capacity and personnel, deal with large numbers of infectious remains, and provide risk communication to the public and the media. Additionally, healthcare facilities and personnel may be involved in epidemiological investigations, contact tracing, and distribution of

mass antibiotic prophylaxis and vaccinations to the community. This chapter reviews some of the legislation and authorizing acts relevant to the response to a biological event, the NRP, the role of the Department of Defense (DoD) in support of civil authorities, and key features of the local response, including disease containment, mass patient care, mass prophylaxis, and mass fatality management.

DoD healthcare providers and planners must be familiar with these concepts because they may be required to provide the medical response on military reservations or in the deployed setting, or they may need to augment the medical response in civilian communities after a natural or artificial biological incident. For example, the military may be called on to “effect a quarantine,” possibly using National Guard troops under federal control in response to an avian influenza outbreak.²

Military medical treatment facilities should maintain an emergency management plan outlining their response to disasters and mass-casualty incidents using an all-hazards approach. These plans should include specific annexes that detail the response to an intentional release of a biological agent and outbreaks of emerging or reemerging infectious diseases.

THE NATIONAL RESPONSE

Legislation

National policy and legislation concerning biological warfare and terrorism provide the foundation for key aspects of the federal response to a biological event. An overview of the pertinent legislation is provided below.

The Stafford Act

The Robert T Stafford Disaster Relief and Emergency Assistance Act³ is the cornerstone legislation for providing federal assistance to states and territories during disasters and emergencies. This act outlines the federal programs available and procedures for disaster preparedness, including mitigation assistance, major disaster and emergency assistance administration, major disaster assistance programs, emergency assistance programs, and emergency preparedness. The Stafford Act provides an orderly and continuing means of assistance by the federal government to state and local governments in carrying out their responsibilities to “alleviate the suffering and damage resulting from disasters” and establishes procedures for states to re-

quest disaster assistance from the federal government. Under this act, a state governor may request that the president declare a major disaster or emergency and direct federal assistance to the state, as long as the disaster is of such severity and magnitude that effective response is beyond the capability of the state.

Defense Against Weapons of Mass Destruction Act

In 1997 Congress enacted the Defense Against Weapons of Mass Destruction Act,⁴ referred to as the Nunn-Lugar-Domenici Act. This act contains initiatives to improve the overall national preparedness for large-scale terrorist attacks of a chemical, biological, radiological, nuclear, or high-yield explosive (CBRNE) nature. Among its provisions is the Domestic Preparedness Program, which provides training, expertise, and equipment grants to the 120 largest US cities. Originally assigned to the DoD and administered by the Soldier’s Biological and Chemical Command (now the Research, Development, and Engineering Command), the Domestic Preparedness Program has provided data on modeling of biological incidents as well as templates and guidelines to assist communities in improving

preparedness for such events (some of these products will be discussed in more detail in the section on local response). The Domestic Preparedness Program was transferred to the Department of Justice, under the Office of Domestic Preparedness, in 2002,⁵ and later to the Department of Homeland Security (DHS).

Emergencies Involving Chemical or Biological Weapons Act

This act allows the attorney general to request DoD assistance directly in response to an emergency involving biological or chemical weapons of mass destruction that exceeds the capability of civilian authorities. This DoD assistance may consist of identifying, monitoring, containing, disabling, or disposing of the weapon, but not direct law enforcement actions.⁶

The Homeland Security Act of 2002

This act established the DHS to prevent terrorist attacks within the United States, reduce the country's vulnerability to terrorism, minimize the damage of and assist in the recovery from terrorist attacks, and act as the focal point for natural and manmade crisis and emergency planning.⁷ The DHS is charged with the following:

- coordinating federal-level preparedness and working with state, local, tribal, parish, and private-sector emergency response providers to combat terrorism;
- consolidating previously existing federal emergency response plans into a single, coordinated NRP;
- ensuring adequate planning, training, and exercise activities;
- conducting risk and vulnerability assessments of critical infrastructure;
- identifying priorities for protection; and
- securing the borders, territorial waters, ports, terminals, waterways, and air, land, and sea transportation systems of the United States.

The National Response Plan

Released in December 2004, the NRP provides a framework for the response to incidents of national significance when the following situations occur (see chapter 20):

- a federal department or agency acting under its own authority requests the assistance of the secretary of Homeland Security;

- an event overwhelms the resources of state and local authorities, and those authorities request federal assistance;
- more than one federal department or agency is substantially involved in responding to an incident; or
- the president has directed the secretary of Homeland Security to assume responsibility for managing a domestic incident.⁸

The NRP integrated previously existing plans, including the initial NRP, the Federal Response Plan, the US Government Interagency Domestic Terrorism Concept of Operations Plan, and the Federal Radiological Emergency Response Plan, to establish a comprehensive, national, all-hazards approach to domestic incident management across a spectrum of activities, including prevention, preparedness, response, and recovery.

The NRP established the National Incident Management System (NIMS) as a standardized approach for managing all major incidents, regardless of etiology, that unifies federal, state, and local lines of government for incident response using the Incident Command System. The Incident Command System standardizes the organization of incident management response by creating five sections: (1) command, (2) operations, (3) planning, (4) logistics, and (5) finance/administration.⁹ NIMS incorporates a unified command structure to ensure coordination and joint decisions on objectives, strategies, plans, priorities, and public communications among different jurisdictions and multiple agencies. A key component of the NRP, NIMS allows several different agencies and organizations to work together with similar command, control, and coordination elements.

The National Response Plan Base Plan and Emergency Support Functions

The NRP is designed to handle incidents at the lowest jurisdictional level possible. The secretary of Homeland Security executes the overall coordination of federal incident management activities, and other federal departments and agencies carry out their incident management and emergency response responsibilities within the NRP's overarching framework.

There are 15 separate emergency support functions (ESFs) that make up the response components of the NRP (listed in Table 19-1). Each ESF has a primary lead agency responsible for implementation and oversight for that aspect of the response, and additional federal agencies provide support to the primary agency. For example, in ESF #10 (Oil and Hazardous

TABLE 19-1
EMERGENCY SUPPORT FUNCTIONS OF THE NATIONAL RESPONSE PLAN

ESF #	ESF Title	ESF Coordinator
1	Transportation Annex	Department of Transportation
2	Communications Annex	Department of Homeland Security/Information Analysis and Infrastructure Protection/National Communications System
3	Public Works and Engineering Annex	Department of Defense/US Army Corps of Engineers
4	Firefighting Annex	Department of Agriculture/Forest Service
5	Emergency Management Annex	Department of Homeland Security/Emergency Preparedness and Response/Federal Emergency Management Agency
6	Mass Care, Housing, and Human Services Annex	Department of Homeland Security/Emergency Preparedness and Response/Federal Emergency Management Agency
7	Resource Support Annex	General Services Administration
8	Public Health and Medical Services Annex	Department of Health and Human Services
9	Urban Search and Rescue Annex	Department of Homeland Security/Emergency Preparedness and Response/Federal Emergency Management Agency
10	Oil and Hazardous Response Annex	Environmental Protection Agency
11	Agriculture and Natural Resources Annex	Department of Agriculture
12	Energy Annex	Department of Energy
13	Public Safety and Security Annex	Department of Homeland Security/ Department of Justice
14	Long-Term Community Recovery and Mitigation Annex	Department of Homeland Security/Emergency Preparedness and Response/Federal Emergency Management Agency
15	External Affairs Annex	Department of Homeland Security

ESF: emergency support function

Material Response Annex), the ESF lead agency and coordinator is the Environmental Protection Agency (EPA), and the US Coast Guard (part of DHS) is a supporting agency. Agencies that provide support for this ESF include the Department of Agriculture, Department of Commerce, DoD, Department of Energy, Department of Health and Human Services (DHHS), DHS, Department of the Interior, Department of Justice, Department of Labor, Department of State, Department of Transportation, General Services Administration, and the Nuclear Regulatory Commission.¹ The ESF modular structure allows mobilization of the precise components that can best address the requirements of the incident. Localized events may be resolved with the activation of a select number of ESFs, whereas some large-scale disasters may require activation of all ESFs.

The federal-level medical response begins with the activation of ESF #8 (Public Health and Medical Services Annex). ESF #8 is coordinated by the secretary of the DHHS principally through the assistant

secretary for public health emergency preparedness. Activation of ESF #8 includes the following core functional areas: (a) assessment of public health/medical needs (including behavioral health), (b) public health surveillance, (c) provision of medical care personnel, and (d) provision of medical equipment and supplies.¹ As lead agency, DHHS coordinates all ESF #8 response actions with its internal departmental policies and procedures.¹⁰ Each support agency is responsible for managing its respective response assets after receiving coordinating instructions from DHHS. ESF #8 response is coordinated through the DHHS secretary's operations center, which maintains frequent communications with the Homeland Security Operations Center.

The National Response Plan Concept of Operations

The secretary of Homeland Security utilizes multi-agency structures at the headquarters, regional, and field levels to coordinate efforts and provide appropri-

ate support to the incident command structure. At the federal headquarters level, incident information sharing, operational planning, and deployment of federal resources are coordinated by the Homeland Security Operations Center, and its component element, the National Response Coordination Center.

Joint Field Office. The multiagency joint field office (JFO), established locally during incidents of national significance, provides a central location for coordination of federal, state, local, tribal, nongovernmental, and private-sector organizations. The JFO’s scalable organizational structure (Figure 19-1) uses the NIMS Incident Command System for managing both preincident and postincident activities. The JFO does not manage on-scene operations; rather, it provides support to on-scene efforts while also conducting broader support operations that may extend beyond the incident site.

The JFO’s coordinating officials include the principal federal official, the federal coordinating officer, the state coordinating officer (appointed by the governor), and other senior federal officials. The federal coordinating officer, who works in partnership with the state coordinating officer and the governor’s authorized representative, conducts an initial appraisal of the assistance most urgently needed and coordinates the timely delivery of federal assistance to affected state, local, and tribal governments and disaster victims. The JFO coordination staff includes the chief of staff, external affairs personnel, Office of the Inspector General personnel, the defense coordinating officer (DCO), the safety coordinator, and liaison officers.

The Defense Coordinating Officer. As the DoD’s single point of contact at the JFO, the DCO coordinates and processes requests for defense support for civil

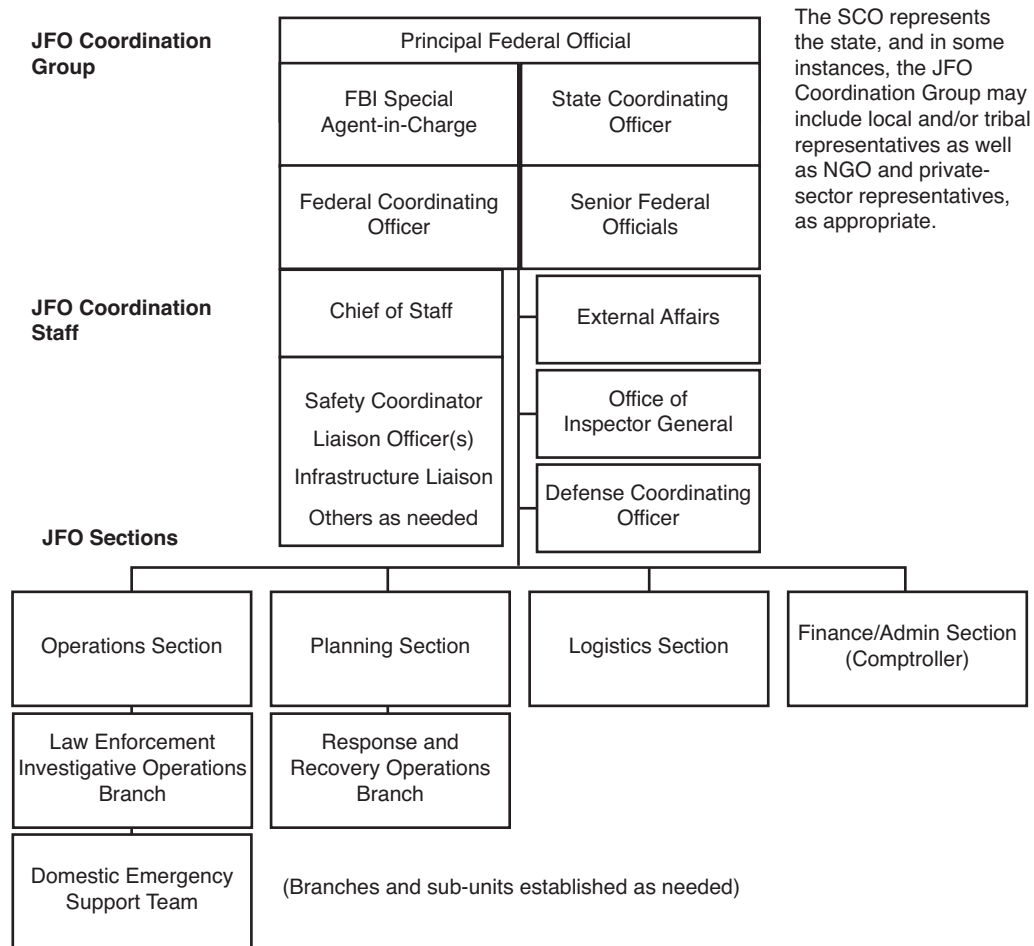


Fig. 19-1. Organizational Structure of the Joint Field Office
 FBI: Federal Bureau of Investigation
 JFO: Joint Field Office
 NGO: nongovernmental organization
 SCO: state coordinating officer

authorities originating at the JFO. The DCO's specific responsibilities include processing requirements for military support, forwarding mission assignments to the appropriate military organizations through DoD-designated channels, and assigning military liaisons, as appropriate, to activated ESFs.

Defense Support to Civilian Authorities

DoD provides defense support for civil authorities in response to requests for assistance during domestic incidents, including terrorist attacks, major disasters, and other emergencies on a reimbursable basis. The initial requests for assistance, usually from the lead or primary agency, are made to the Office of the Secretary of Defense, Executive Secretariat. If the secretary of defense approves the request, DoD designates a supported combatant commander to lead the response. The commander determines the appropriate level of command and control, usually deploying a senior military officer to the incident site. Under most circumstances, the senior military officer at the site is the DCO. The commander may also use a joint task force to consolidate and manage supporting military activities. The joint task force commander exercises operational control of all allocated DoD resources (however, neither the joint task force commander nor the DCO handle US Army Corps of Engineers resources, National Guard forces operating in state active duty or Title 32 status, or, in some circumstances, DoD forces in support of the Federal Bureau of Investigation).

Defense Department Medical Response Support

DHHS, the lead agency for the federal medical response, may request assistance from the DoD (operating under ESF #1, Transportation, and ESF #8). This assistance may include the following:

- activating the DoD National Disaster Medical System (NDMS) patient reception plans, which manage medical evacuation of seriously ill or injured patients from a collection point in or near the incident site to locations where hospital care or outpatient services are available (such as nearby NDMS nonfederal hospitals, Veterans Administration hospitals, and DoD military treatment facilities);
- deploying military medical personnel (including reserve and National Guard medical units) to provide triage, medical treatment, and mental health support, as well as public health protection (such as assistance with

food, water, wastewater, solid waste disposal, vectors, hygiene, and other environmental conditions);

- providing available DoD medical supplies, including blood products, for distribution to medical care locations;
- providing services such as evaluations, risk management appraisals, and confirmatory laboratory testing support; and
- assisting in the management of human remains, including victim identification and mortuary affairs.

Other Defense Department Support

Support for law enforcement and domestic counterterrorism activities may be provided in limited circumstances consistent with applicable laws and, in some circumstances, independent of the DCO. Imminently serious conditions resulting from any civil emergency may require immediate action to save lives, prevent human suffering, or mitigate property damage. When time does not permit approval from headquarters in such situations, local military commanders and responsible DoD officials are authorized by DoD directive¹¹ and preapproval by the secretary of defense, subject to any supplemental direction from their DoD component, to respond to requests from civil authorities consistent with the Posse Comitatus Act, referred to as "immediate response."

In addition to direct support for incident response, DoD possesses specialized capabilities that may be requested (in addition to the medical services described above), including use of test and evaluation facilities and capabilities; education and exercise expertise; explosive detection; technical escort; and the transfer of applicable technologies, including those developed through DoD science and technology programs. The DoD Homeland Defense Coordination Office, established at DHS headquarters, facilitates interdepartmental cooperation and transfer of these capabilities to the emergency responder community.

The Biological Incident Annex

The all-hazards approach is a consistent theme throughout the NRP; however, response to an intentional biological agent release may entail additional consequence management actions. A coordinated response of several federal agencies is the key to successful consequence management. Over 40 federal departments and agencies have some role in combating terrorism, and over 20 departments and agencies participate in preparations for or responses to the public

health and medical consequences of a bioterrorist attack.¹² The NRP Biological Incident Annex identifies the actions and coordination needed in response to the intentional release of a biological agent.

DHHS is the primary federal agency for the public health and medical preparation for and response to a biological terrorism attack, as well as a naturally occurring outbreak from a known or novel pathogen, including an emerging infectious disease. Per the NRP, state and local governments are primarily responsible for detecting and responding to disease outbreaks and implementing measures to minimize an outbreak's health, social, and economic consequences. Whereas DHHS coordinates the overall federal public health and medical emergency response efforts, DHS coordinates the overall nonmedical federal support and response actions.

The NRP Biological Incident Annex identifies the following key elements of an effective biological response:

- rapid detection of the outbreak;
- swift agent identification and confirmation;
- identification of the population at risk;
- determination of how the agent is transmitted, including an assessment of the efficiency of transmission;
- determination of susceptibility of the pathogen to treatment;
- definition of the public health, medical, and mental health implications;
- control and containment of the epidemic;
- decontamination of individuals, if necessary;
- identification of the law enforcement implications of the threat;
- augmentation of local health and medical resources;
- protection of the population through appropriate public health and medical actions;
- dissemination of information to enlist public support;
- assessment of environmental contamination and cleanup or decontamination of biological agents that persist in the environment; and
- tracking and preventing secondary or additional disease outbreak.¹

Once notified of a threat or disease outbreak that may require significant federal public health or medical assistance, DHHS convenes a meeting of all organizations involved in ESF #8. The immediate tasks are to identify the population affected, the population at risk, and the geographic scope of the incident. The initial public health and medical response includes some or all of the following actions:

- targeted epidemiological investigation;
- intensified surveillance in healthcare settings for patients with certain clinical signs and symptoms;
- intensified collection and review of potentially related information; and
- organization of federal public health and medical response assets including personnel, medical supplies, and materiel.

The public health system, starting at the local level, is required to initiate appropriate protective and responsive measures for the affected population, including deploying first responders and other workers engaged in incident-related activities. These measures may include mass vaccination or prophylaxis for populations at risk, including those who might be exposed from secondary transmission or the environment. The overarching goal is to develop a prioritized list of treatment recommendations based on epidemiological risk assessment, the biology of the disease or agent in question, and the deployment of the strategic national stockpile (SNS) as soon as possible (see below for a discussion of the SNS).

DHHS and partner organizations evaluate the incident and make recommendations to the appropriate public health and medical authorities on the need for quarantine, shelter-in-place, or isolation to prevent the spread of disease. DHHS works closely with DHS when recommending the use of NDMS or the US Public Health Service Commissioned Corps. The governors of affected states implement isolation or social-distancing requirements using state and local legal authority, and DHHS may take federal action to prevent the interstate spread of disease. State and local authorities also assist with the implementation and enforcement of isolation and quarantine actions. The scope and nature of the outbreak may require mass isolation or quarantine of affected or potentially affected persons, as well as food, animals, and other agricultural products.

Defense Department Bioterrorism Response Assets

In addition to providing medical care, logistics, and evacuation, the DoD maintains several specialized organizations, equipment, and capabilities to respond quickly to an intentional biological agent release. For example, the US Army Medical Research Institute of Infectious Diseases (USAMRIID) performed approximately 19,000 anthrax assays within a short period immediately after the anthrax mailings in 2001.¹³ The following is an overview of some of these organizations and their interactions.

US Northern Command

The US Northern Command (NORTHCOM) was established in 2002 to plan, organize, and execute homeland defense and civil support missions. Several joint task forces have been assigned to NORTHCOM, including Joint Task Force North, Joint Force Headquarters National Capital Region, Joint Task Force Alaska, and Joint Task Force Civil Support. NORTHCOM's civil support capabilities include domestic disaster relief operations for fires, hurricanes, floods, and earthquakes; counter-drug operations; and consequence management assistance for events such as a terrorist's use of a weapon of mass destruction.¹⁴

Joint Task Force Civil Support is headquartered at Fort Monroe in Hampton, Virginia, and consists of active-duty, National Guard, and reserve military members of all service branches, as well as civilian personnel commanded by a federalized National Guard general officer. When approved by the secretary of defense and directed by the NORTHCOM commander, Joint Task Force Civil Support deploys to a CBRNE incident site in the United States and its territories and possessions. At the site, the task force executes command and control of designated DoD forces and provides support to the civil and federal authorities managing the incident to save lives, prevent injury, and provide temporary critical life support.¹⁵

20th Support Command

Whereas NORTHCOM operates within the United States, the 20th Support Command works outside the country, serving as a command and control element and provider of US CBRNE operational response teams and technical augmentation cells worldwide. Also called the CBRNE Command, it is subordinate to the US Army Forces Command. The 20th Support Command brings command and control of the Army's specialized weapons of mass destruction operational response assets together to provide a single point of contact when a coordinated response to the threat or use of weapons of mass destruction is needed anywhere in the world. Its mission is to command and control organic and allocated Army technical assets to support full-spectrum CBRNE technical operations that detect, identify, assess, render safe, dismantle, transfer, and dispose of CBRNE incident devices and materiel, including unexploded ordnance and improvised explosive devices.

The 20th Support Command also provides CBRNE technical advice and expertise within the United States, to help mitigate incidents involving the nation's chemical warfare stockpile, manage recovery and disposal

of legacy chemical and biological munitions and materials from formerly used defense sites, and conduct technical escort of chemical surety materiel in support of the management of chemical stockpile and chemical defense research and development. This unit has the technical expertise to conduct sensitive site exploitation, disablement, disposition, demilitarization, and consequence management operations. It also augments and reinforces installation support teams after a CBRNE incident at any US Army facility, and supports other CBRNE response missions as directed by the commander of the US Army Forces Command.¹⁶

Weapons of Mass Destruction Civil Support Teams

The Weapons of Mass Destruction Civil Support Teams were established to provide rapidly deployed federal assistance to local authorities at incident sites. They are composed of 22 full-time National Guard members (either Army or Air National Guard), who are federally resourced, trained, and exercised. If the teams are federalized, they fall under Joint Task Force Civil Support operational command and control. Teams are designed to be ready to deploy within 4 hours to anywhere within their area of responsibility, with their own detection and decontamination equipment, medical supplies, and protective gear. Each team has two large pieces of equipment: (1) a mobile analytical laboratory for field analysis of chemical or biological agents and (2) a uniform command suite to provide interoperability of communications to all responders. The teams provide assistance by identifying agents and substances, assessing current and projected consequences, advising on response measures, and assisting with requests for additional military support. Their role can include entering a contaminated area to gather air, soil, and other samples for on-site evaluation.^{17,18}

Chemical and Biological Incident Response Force

Located 26 miles from Washington, DC, the Chemical and Biological Incident Response Force was formed in 1996 and consists of marines and sailors who can forward-deploy or respond to a credible threat of a CBRNE incident. The force assists local, state, or federal agencies and unified combat commanders in consequence management operations by providing capabilities for agent detection and identification; casualty search, rescue, and personnel decontamination; and emergency medical care and stabilization of contaminated personnel. In addition to several exercises, the force has demonstrated its capabilities in the 2001 anthrax and 2004 ricin decontamination operations of the US Senate office buildings.¹⁹

US Army Medical Research Institute of Infectious Diseases' Patient Containment Care Suite

Maximum biological containment consists of four principal features: (1) a physical protective barrier, (2) an air pressure barrier, (3) a filtered inflow and outflow air supply, and (4) waste disinfection.²⁰ In the United Kingdom, containment care is provided by a negative-pressure, polyvinylchloride envelope isolator, similar to the reverse-barrier isolators used historically to protect patients with profound immunodeficiency disorders. USAMRIID has a two-bed containment care unit specifically engineered to provide these features.

The Centers for Disease Control and Prevention (CDC) categorizes the laboratory safety requirements of potentially pathological agents into one of four categories based on pathogenicity, potential for aerosol transmission, and whether an effective vaccine or therapy exists. A laboratory's biosafety level (BSL) is determined by its available safety controls relating to practices, techniques, and containment fixtures and facilities. Agents that require BSL-4 laboratory procedures include filoviruses, arboviruses, arenaviruses, hantaviruses, the severe acute respiratory syndrome (SARS) virus, new influenza strains, and other viruses with a high or unknown risk of aerosol transmission. The BSL terminology can also be applied to hospitals, which, in addition to handling specimens in their clinical laboratories, care for infectious patients.

USAMRIID's patient-containment care suite has conditions analogous to a BSL-4 laboratory. During operation, the doors to the unit are sealed with duct tape and the interior pressure is brought to 0.5 inches of water negative pressure, corresponding to 18 air exchanges per hour. Air entering the suite passes through a high-efficiency particulate air (HEPA) filter and exhausted air passes through double HEPA filtration.

Individuals working in the unit wear protective Chemtursion encapsulation suits (ILC Dover, Frederica, Del), which connect to hoses providing overpressure and a clean air supply (Figure 19-2). Air entering the suits through these hoses has passed through both charcoal and HEPA filters. Personnel enter and exit through an anteroom, where they don the protective suits, and then pass through a decontamination shower with double-sealed closure doors. The chemical disinfectant shower consists of a 1-minute water rinse, followed by 2.5-minute chemical shower with a 5% solution of Micro-Chem Plus (National Chemical Laboratories, Inc, Philadelphia, Pa) ammonium compound, followed by another 1-minute water rinse.

Materials can pass in and out of the unit through one of four pathways. Sewage passes through dedicated lines to a steam treatment plant where all of the sew-

age waste is sterilized. Solid waste is passed through a pass-through autoclave. Food and medications are passed through an ultraviolet light box, and clinical specimens are sealed in plastic bags and passed through a chemical dunk tank.

The two patient rooms have standard intensive care monitoring equipment comparable to any medical center intensive care unit. They are staffed by a team of intensive care medical personnel from Walter Reed Army Medical Center, consisting of doctors, nurses, and ancillary support personnel. The team trains in the facility on a quarterly basis to provide the full range of hospital services that a medical intensive care unit patient may need.²¹

Since its construction in 1972, 17 patients have been admitted to the unit, all of whom were research scientists with occupational exposure to BSL-3 or BSL-4 agents. If a patient admitted to the unit were exposed to an agent with a high or unknown risk of aerosol transmission, particularly a highly lethal agent, consideration must be given to postexposure isolation prior to onset of illness. Although none of the admissions resulted in active disease, the most recently admitted patient, a scientist exposed to the Zaire strain of the Ebola virus in March 2004, was kept in isolation in the unit for 3 weeks while being tested daily for infection.

The major limitation of USAMRIID's containment care unit is the lack of continuous on-site medical-center-level support for patients who become critically ill. Laboratory services, other than specific agent-related testing performed by USAMRIID's diagnostic systems division, require outside support for all but a few basic procedures. Studies performed in unre-



Fig. 19-2. Provision of medical care under biosafety level 4 conditions in the US Army Medical Research Institute of Infectious Diseases' patient containment care suite.

denial laboratories for human clinical use require the review of a clinical pathologist before they can be used to make treatment decisions. Simple radiographs can be performed in the USAMRIID unit, but they

require evaluation from outside organizations. The unit's greatest limitation, however, involves training personnel to provide care under the constraints of high-level containment.

THE LOCAL RESPONSE

Initial Response to a Biological Event

Biological agents may be attractive weapons for terrorists for several reasons: (a) some agents have a high case-fatality rate; (b) some agents are contagious and may propagate secondary infections throughout the community; (c) the psychological impacts of a bioterrorism event can cause a far greater effect than the agent alone; and (d) because casualties from a covert release of a biological agent will not likely be identified until patients develop symptoms after the disease incubation period, the perpetrator has time to leave the scene.²² An intentional biological event may not be detected until several days or weeks after the incident, and the first clues will likely be an increase in emergency department and clinic evaluations for nonspecific influenza-like symptoms. As patients develop more specific symptoms, astute clinicians may make the presumptive diagnosis of an intentional agent exposure.

Healthcare facilities, clinical laboratories, public health officials, law enforcement, and civil authorities need to work together to create plans for responding to bioterrorism events. Military healthcare facilities need to work closely with the local civilian community to set up mutual aid agreements and memoranda of understanding in the event a bioterrorism event occurs either on or off the military installation. Figure 19-3 depicts a sample response to an intentional biological agent event.

Containment

Active containment of disease is a pillar of outbreak management. Epidemiological evaluations and active disease surveillance will help identify people who have been exposed to the initial biological agent release, the active cases of disease, and in the case of communicable diseases, contacts of those with active disease. Biological events may be overt or covert and, unlike chemical, nuclear, and high-explosive events, biological agent aerosols are odorless, colorless, and may not cause obvious symptoms for several days or longer, depending on the incubation period of the organism.

Overt biological attacks may be recognized if the attack is announced before the release, the attack is

witnessed, or responsibility is claimed immediately after an initially unrecognized agent release. Educating the public, first responders, and healthcare providers is necessary to increase awareness and recognition of overt attacks. Several organizations will be involved with an on-scene response to an overt biological attack, including the fire department, hazardous materials teams, emergency medical services (EMS), and police. On-scene tasks include the need to secure the scene; identify those who have been exposed; decontaminate patients, equipment, and the environment; and initiate both criminal and epidemiological investigations. Those who have been exposed or are likely to be exposed should be evaluated for prophylaxis, depending on the biological agent suspected. Demographic data should be collected on everyone at the scene so that adequate follow-up and evaluation can be performed. People with gross contamination need to be decontaminated. On-scene response procedures, training, personal protective equipment (PPE), and medical surveillance are governed by the Occupational Safety and Health Administration regulation, "Hazardous Waste and Operations and Emergency Response."²³

The incident site will likely be sectioned into different zones by the incident commander to decrease the spread of contamination, control the number of personnel authorized in the high-risk areas, and delineate required levels of personal protection to be worn. Traditionally, incident scenes will have at least three zones: (1) hot zone (contaminated area); (2) warm zone (the area where decontamination of personnel and equipment occurs); and (3) the cold zone (the uncontaminated area where workers should not be exposed to hazardous conditions). Despite debate over the role of medical personnel entering the "hot" or "warm" zone, emergency medical personnel need to be trained in scene safety, PPE, and standard operating procedures for on-site response. EMS responders should use PPE as specified by the incident commander. Minimal PPE that should be carried or immediately available to EMS workers includes eye protection; a single-use barrier garment, such as Tyvek (DuPont, Wilmington, Del); hooded chemical-resistant clothing; nitrile gloves; chemical-resistant footwear covers; properly fit-tested N100 or N95 masks; and an escape hood to allow workers to remove themselves from contaminated areas.²⁴ A full-face piece respirator with a P100

filter or powered air-purifying respirator with HEPA filters may be used when it can be determined that an aerosol-generating device was not used to create high airborne concentrations or when dissemination was by a letter or package that was easily bagged.²⁵ If medical personnel are to provide medical treatment including triage during decontamination, a minimum of level C PPE should be worn, including a hooded, powered, air-purifying respirator with a protection factor of at least 1,000, with an appropriate filter, and chemical-resistant gloves, boots, and suits to match or exceed the level of respiratory protection worn.²⁶ Recommendations for PPE in the hot zone include the use of pressure-demand, self-contained breathing apparatus approved by the National Institute for Occupational Safety and Health (NIOSH), in conjunction with either level B or

level A protective suits. Level B suits should be worn if the suspected biological aerosol is no longer being generated or a splash hazard may be present. Level A suits should be worn when responding to a suspected biological incident in which the type of airborne agent is unknown, the dissemination method is unknown, dissemination via an aerosol-generating device is still occurring, or dissemination via an aerosol-generating device has stopped, but no information is available on the duration of dissemination or concentration of exposure.²⁵

The need to decontaminate people exposed at the incident site varies based on the agent released (if known), the method of dissemination, and the individual's potential for exposure. Agents that are released completely as aerosols behave as a gas and leave little

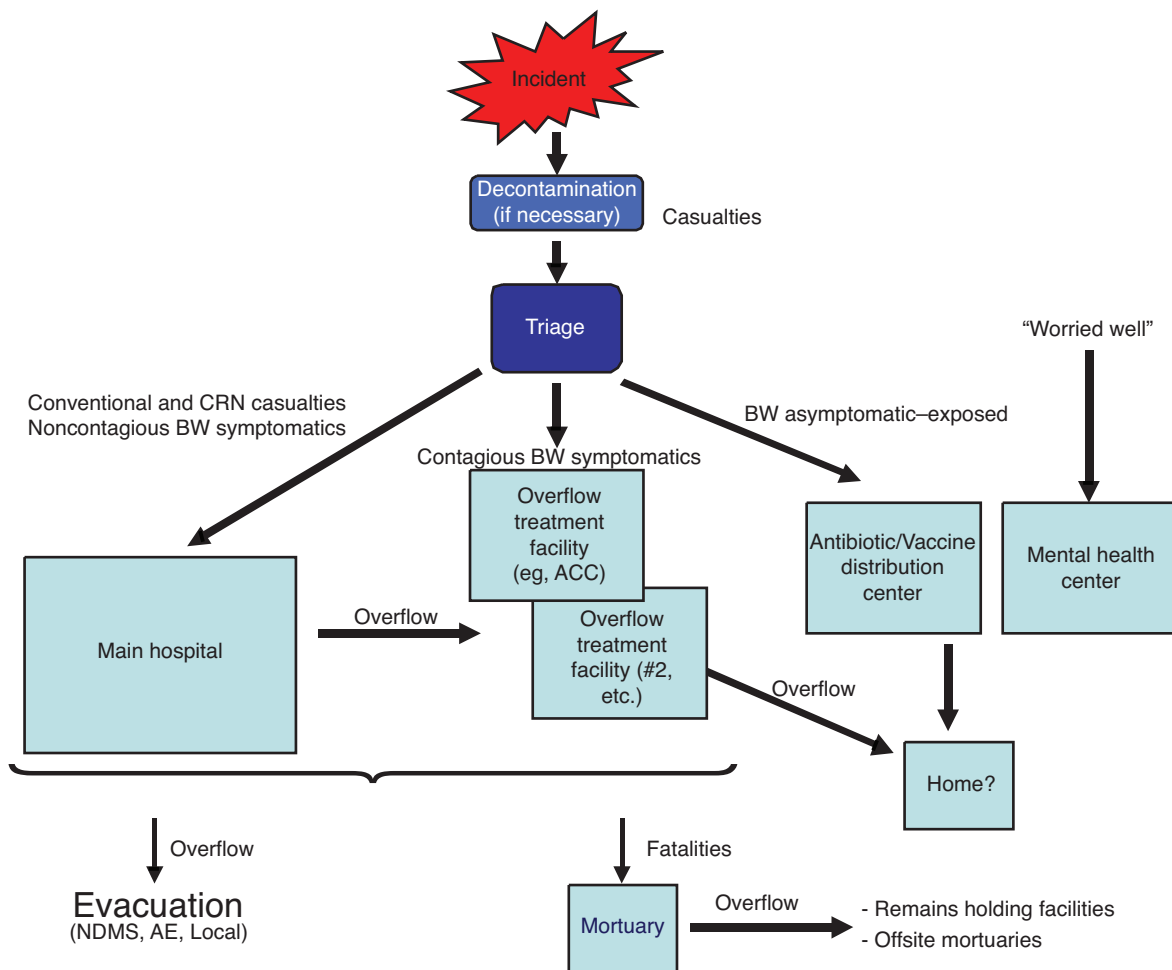


Fig. 19-3. Response to an intentional biological agent flow diagram

ACC: acute care center
 BW: biowarfare
 CRN: chemical, radiological, nuclear
 NDMS: National Disaster Medical System

to no residual environmental contamination. Any gross contamination from dry powders or liquid agents requires decontamination. Personnel decontamination should be accomplished with high-volume, low-pressure water at a minimum of 60 pounds per square inch with a decontamination solution including soap, water, and hypochlorite, or a variety of commercially available dry, gelled, or powdered absorbents.²¹ For most biological agents, showering with soap and water is the only necessary decontamination.

For certain types of biological incidents, especially anthrax spores, it may be necessary to assess the extent of contamination and to decontaminate victims, responders, animals, equipment, buildings, critical infrastructure, and large outdoor areas. Additionally, powdered agents may lend themselves to secondary aerosolization (created by kinetic energy near the settled powder). One study has shown that a person actively performing exercise for 3 hours on an area of ground contaminated with 2×10^7 /m² of *Bacillus subtilis* spores would inhale between 1,000 and 15,000 spores.²⁷ Secondary aerosolization may pose a significant problem when dry agent is released in an enclosed environment. Before decontamination of the Hart Senate office building, agar plates were placed in an office and normal activity was simulated. Sixteen of 17 agar plates subsequently grew *B anthracis* colonies.²⁸

Under the Federal Insecticide, Fungicide, and Rodenticide Act,²⁹ decontamination solutions must be registered with the EPA. No decontamination chemicals for use against biological agents have been approved by the EPA, although a review of current technologies from more than 75 different vendors is being conducted.³⁰ Responders to an incident site must request an emergency exemption from the EPA for each specific event before chemicals can be used for biological decontamination. The emergency exemption allows the sale, distribution, and use of an unregistered pesticide for a limited time. The three broad categories of decontamination technologies are (1) liquid-based topical agents, such as hypochlorite; (2) foams and gels; and (3) gaseous and vapor technologies (fumigants), including chlorine dioxide gas, vapor-phase hydrogen peroxide, paraformaldehyde, and methyl bromide. No single technology is applicable in all situations. In general, liquids are effective cleaners of nonporous surfaces, but they can cause surface corrosion or degradation. Foams and gels have shown some promising results, but they present postdecontamination cleanup problems. Gases and vapors are effective in destroying biological contamination under controlled conditions (eg, in sterilization chambers) and, in some cases, in field

remediation, but they warrant further evaluation for use in large buildings.³¹ Chemicals that have been granted crisis exemptions by the EPA for biological agent decontamination include chlorine dioxide, ethylene oxide, hydrogen peroxide, hypochlorite, and paraformaldehyde.³²

A large covert release of a biological agent represents a public health catastrophe that could involve tens of thousands of victims and rapidly overwhelm local resources. For example, a 1970 report from the World Health Organization predicted the number of casualties and fatalities from various agents delivered as aerosols from an aircraft over a 2-kilometer line near a population center of 500,000. Released anthrax would disseminate over 20 kilometers, causing 125,000 casualties and 95,000 deaths.³³ Patients may not develop symptoms for several days (depending on the incubation period of the agent), may be dispersed over a large geographic area, and may unwittingly infect others if a contagious agent has been released. Recognition of the attack occurs when sick patients present to medical clinics and emergency departments. In this situation, the healthcare facility is the frontline of the response, rather than the response teams typically at the scene of a catastrophic event.

Covert attacks may be recognized through surveillance if the number of symptomatic casualties is large. A significant aerosol attack will likely cause a dramatic increase in patients presenting with nonspecific constitutional symptoms, which may be noted anecdotally by clinicians, or by public health officials and epidemiologists conducting active surveillance. Laboratories or pharmacies may also note an unusual pattern of findings. Attacks with agents that are not contagious or infect only a handful of patients would probably not be detected by surveillance. This type of attack might be recognized by astute clinicians³⁴; however, healthcare providers may fail to include biological warfare or terrorist agents in the differential diagnosis of casualties. The 2003 report of the Gilmore panel, a government-funded advisory group assessing terrorism response capabilities, concluded that the level of expertise in recognizing and dealing with a terrorist attack involving a biological or chemical agent is problematic in many hospitals.³⁵ Well-trained, astute clinicians familiar with biological terrorism agents and their manifestations would provide the earliest possible detection of a covert biological attack; however, such training must be significantly increased.³⁶

A large biological agent attack will likely extend beyond the boundaries of a single community, with contagion spread by commuters and other travelers. An event at a military facility will affect the public health of the surrounding community, and airports in

an affected area could facilitate the spread of disease to other parts of the United States and the world. The nature of a covert attack with biological organisms is likely to produce widespread fear that may present unique challenges to responders, government officials, and the public.³⁷

Isolation and Quarantine

The initial response in most biological terrorism drills is to restrict movement, cordon off the area, and enforce quarantine of the population. Although the terms “isolation” and “quarantine” are used somewhat interchangeably, there are distinct differences between the two. Isolation is the separation and confinement of ill individuals known to be or suspected of being infected with a contagious disease to prevent them from infecting others. Quarantine is the compulsory physical separation, including restriction of movement, of populations or groups of healthy individuals who have potentially been exposed to a contagious disease. Quarantine may be voluntary or mandated, and state laws determine the specific mechanisms of instituting the quarantine, its duration, and its enforcement.³⁸

The authority for isolation and quarantine comes from the Public Health Service Act,³⁹ which gives the secretary of DHHS the responsibility to prevent introduction, transmission, and spread of communicable diseases. The diseases covered under this act must be specified by executive order of the president, on recommendation of DHHS. The federal government is concerned with preventing introduction of communicable diseases into the country. States have been given the authority to declare and enforce quarantine within their borders. The state health director may have this authority, or it may be delegated to the local health director. In addition to the legal considerations of authority and enforcement of quarantine, several other factors may influence quarantine adherence. During the SARS outbreak in 2003, several Asian countries instituted quarantine of large numbers of people: approximately 130,000 people in Taiwan; 23,000 to 30,000 people in Toronto, Canada⁴⁰; and roughly 7,800 people in Singapore were placed in quarantine either at home or in a designated facility.⁴¹ The decision to impose quarantine includes the following considerations: (a) Do the public health and medical analyses of the situation warrant the imposition of quarantine? (b) Are the implementation and maintenance of a large-scale quarantine feasible? (c) Do the benefits of a large-scale quarantine outweigh the possible adverse consequences (economic impact, perceptions of ethnic bias, government mistrust, and potential for increased risk of disease transmission in those quarantined together)?⁴²

DoD medical treatment facilities must be aware of the quarantine laws in their respective states. Although commanders have authority over their soldiers, sailors, airmen, and marines, a significant number of dependents may reside outside the military reservation and fall under the state’s quarantine laws.

Mass Patient Care

Healthcare needs during a large-scale bioterrorism event can quickly overwhelm medical facilities. Mitigation strategies include streamlining the facility logistical system, creating facility and local stockpiles of anticipated medications, and establishing plans for reception and distribution of the SNS. Communities must be able to expand both prehospital and hospital capacity. Hospital and community plans to resource patient care on a grand scale need to be realistic, known, and practiced.

Prehospital Transport

During a large-scale bioterrorism event, infected casualties and the “worried well” who seek aid will likely overwhelm emergency medical services and hospitals.⁴³ In an overt attack, casualties from conventional injuries (eg, blast or orthopedic injuries from explosions) or those with exacerbations of preexisting chronic diseases (eg, asthma) may need transport to a healthcare facility by EMS. Personnel at the incident site who have been exposed may become infected, but are not contagious, and should not develop symptoms until completion of an incubation period that varies depending on the specific agent involved.

During the sarin nerve agent attack in Tokyo, approximately 5,000 to 6,000 persons were exposed. Of those exposed, 3,227 sought medical care, and 493 were admitted to 41 hospitals.⁴⁴ Many of these patients arrived by commercial transportation or privately owned conveyance rather than by EMS. It can be estimated that approximately half of the patients from a large-scale terrorism event will arrive by EMS within 1 to 2 hours.²⁴ Therefore, local and regional medical resources must be available within the first few hours. Hospitals must be prepared to evaluate patients for exposure and gross contamination before allowing them into the facility. Plans for both prehospital and hospital “surge capacity” should be in place and exercised before an incident occurs.

After a covert event, the EMS may be quickly overwhelmed with transport of sick patients. Although the event may not be suspected at the time, supervisors may see an increase in transports for nonspecific or unusual complaints that coincides with the incubation

period of the agent released. In New York, the onset of the annual influenza season is recognized by monitoring EMS transports.⁴⁵ In the event of a large-scale aerosol release of a biological agent, potentially infected individuals will likely not be grossly contaminated because they will have changed clothes and showered since the event. Depending on the agent released, these patients may be contagious. Standard precautions should be used for all patients, including the use of surgical masks for those with pulmonary involvement. EMS personnel are at risk of contracting contagious diseases from transporting ill patients and need to protect themselves accordingly. Infection of EMS personnel can devastate the prehospital system. During the SARS outbreak, Toronto's 850 paramedics had 1,166 potential SARS exposures, requiring 436 of them to be placed in 10-day home quarantine (staying isolated from other persons within the home, continuously wearing an N95 respirator, and taking their temperatures twice a day). SARS-like illnesses developed in 62 of the paramedics, and suspected or probable SARS required the hospitalization of four others. When the outbreak's second phase began, more than 200 paramedics had contact with patients with SARS and were quarantined.⁴⁶

Hospital Triage

Traditional triage systems seek to establish a small number of categories among victims that indicate the urgency with which they should be treated. The adequacy of the triage system used in large-scale events depends on many variables, including the nature of the event, the population affected, and the competence of the triage physician. Triage during outbreaks of easily transmitted diseases needs to be based on epidemiology as well as the patient's clinical condition. Epidemiological approaches to triage, considered more appropriate for biological events, sort infected patients into three categories: (1) susceptible individuals, (2) infected individuals, and (3) removed individuals (by successful vaccination, recovery, or death).⁴⁷ An expansion of this system into five categories has been suggested: (1) susceptible individuals (including those with incomplete or unsuccessful vaccination); (2) exposed individuals (those who are infected but are in the incubation period and are noncontagious); (3) infectious individuals (who are symptomatic and contagious); (4) removed individuals (those who have survived and are no longer contagious); and (5) successfully vaccinated individuals (with a confirmed clinical "take" or completed vaccination series).⁴⁸

The goals of triage in this situation are to distinguish individuals who are contagious from those who are

not, and to protect healthcare personnel, other patients, and the community from spread of the disease. The triage center must be able to identify those requiring decontamination immediately after an overt event, including self-referrals. Occupational Safety and Health Administration guidance for first receivers ("healthcare workers at a hospital receiving contaminated victims for treatment") states that minimal PPE for employees in the hospital decontamination zone when the agent is unknown includes the use of a NIOSH-approved, powered air-purifying respirator that provides a protection factor of 1,000; combination NIOSH-approved 99.97% HEPA/organic vapor/acid gas respirator cartridges; double-layer protective gloves; a chemical-resistant suit with openings sealed by tape, a head covering, and eye/face protection (if not part of the respirator); and chemical-protective boots.⁴⁹

Response to a covert event with a contagious agent may require moving the triage site away from the patient-care facility to prevent nosocomial spread of the disease. Occupational Safety and Health Administration first-receiver guidance for employees outside the hospital decontamination zone includes normal work clothes and PPE as appropriate for infection-control purposes. Respiratory precautions should be instituted at the triage center for diseases transmissible by the respiratory route. All persons with signs and symptoms of a respiratory infection, regardless of presumed cause, should be instructed to (a) cover the nose and mouth when coughing or sneezing, (b) use tissues to contain respiratory secretions, (c) dispose of tissues in the nearest waste receptacle after use, and (d) use hand hygiene after contact with respiratory secretions and contaminated objects. In addition to tissues and receptacles for disposal, healthcare facilities should provide conveniently located dispensers of alcohol-based hand rubs as well as soap and disposable towels where sinks are available. Procedure masks (ie, with ear loops) or surgical masks (ie, with ties) may be used to contain respiratory secretions. People who are coughing should sit at least 3 feet away from others in common waiting areas.⁵⁰ These recommendations should be instituted during normal daily triage procedures to assist in decreasing nosocomial transmission of any respiratory disease and to increase staff familiarity with the recommendations before an emergency situation.

Hospital Infection Control

Standard precautions include hand washing, gloves, masks, eye protection, face shields, and gowns when there is a potential for exposure to blood; all body fluids, secretions, and excretions other than sweat, regardless

of whether they contain visible blood; nonintact skin; or mucous membranes. Additional precautions may be needed based on the mechanism of disease transmission. Transmission-based precautions (airborne precautions, droplet precautions, and contact precautions) are designed for patients documented or suspected to be infected with certain highly transmissible or epidemiologically important pathogens. Patients with smallpox require the addition of airborne and contact precautions, patients with pneumonic plague require the addition of droplet precautions, and patients with viral hemorrhagic fevers require the addition of contact and airborne precautions. Casualties from an intentional release of inhalational anthrax, brucellosis, tularemia, equine encephalitis, and toxins (eg, botulinum toxins, ricin, staphylococcal enterotoxin B, and tricothecene mycotoxins) are not contagious and pose no threat of nosocomial spread.

In small-scale events, routine patient placement and infection-control practices should be followed in the facility. However, when the number of patients presenting to a healthcare facility is too large to allow routine triage and isolation strategies, a practical alternative is cohorting patients who present with similar syndromes (ie, grouping affected patients in a designated area).⁵¹

Expanding Surge Capacity

Healthcare systems must have the ability to expand both inpatient and outpatient capabilities during an outbreak or large-scale attack. The amount of surge capacity needed depends on the agent released, the method of dissemination, the number of people exposed, and assessment of the population at risk for both primary and secondary infections. In addition to cohorting, strategies to increase capacity of healthcare systems include the transfer of noncontagious inpatients to other facilities, the transfer of contagious casualties that exceed the healthcare system's capacity to other facilities, and the expansion of the system to include nonhealthcare facilities that may be amenable to patient care. The DHHS Health Resources and Services Administration benchmarks for hospitals in the National Bioterrorism Hospital Preparedness Program include (a) developing adequate portable or fixed decontamination systems for 500 patients per million persons in the population; (b) developing systems that, at a minimum, can provide triage, treatment, and initial stabilization for 500 patients (per million persons in the population) with symptoms of acute infectious disease (especially smallpox, anthrax, plague, tularemia, and influenza) above the current daily staffed bed capacity within 3 hours of a terrorism incident; (c) having the

capacity to maintain at least one suspected case of a highly infectious disease in negative-pressure isolation; and (d) identifying at least one healthcare facility in each region that is able to support the initial evaluation and treatment of at least 10 adult and pediatric patients in negative-pressure isolation within 3 hours postevent.⁵²

Although the actual capacity needed for a biological attack is unknown, it is advantageous for healthcare organizations, local communities, and regional emergency operations planners to identify which assets are available, which assets can be leveraged through mutual aid agreements and memoranda of understanding, and which assets can establish a trigger to request assistance from the state and federal government. Planning for surge capacity allows a structured response to epidemics and pandemics of emergency or reemerging diseases that may overwhelm the healthcare infrastructure, such as the 1918–1919 influenza pandemic, which sickened approximately half of the world's population (1 billion people) and killed 21 million to 40 million people.⁵³

Patient transfers. Mutual aid agreements may include options to transfer patients between facilities, either moving contagious patients between facilities or noncontagious patients out of the affected hospital to make room for contagious patients. Receiving facilities may include local civilian hospitals, military treatment facilities if memoranda of agreement are in place, or NDMS hospitals if the NRP is activated.

Transporting contagious casualties is safe provided appropriate standard and agent-specific transmission precautions are maintained. During the SARS outbreak in Ontario, Canada, a medically based command, control, and tracking center for all interfacility (including acute and long-term care) patient transfers was implemented. The center successfully handled more than 500 transfer requests per day within 36 hours of operation, and more than 1,100 requests per day within 2 weeks. There was no reported spread of SARS resulting from the transfers, and anecdotal evidence demonstrated that the program identified up to 13 new SARS cases.⁵⁴

Isolation wards and cohorting. Additional patient-care space can be obtained by the creation of isolation wards to allow cohorting of patients with the same disease, which may be useful if all negative-pressure isolation rooms are used during a contagious disease outbreak. Designated cohort sites should be chosen in advance in consultation with facility engineering staff, based on patterns of airflow and ventilation, availability of adequate plumbing and waste disposal, and capacity to safely hold large numbers of patients. The cohort site should have controlled entry to minimize

the possibility of transmission to other patients and staff members; however, reasonable access to vital diagnostic services such as a radiology department should be maintained. Critical evaluation of the heating, ventilating, and air conditioning system is needed to limit the possibility of agents spread by aerosol.

Cohorting was used in Canada during the SARS outbreak to isolate 70 exposed patients in three open-plan wards. Elective isolation was carried out immediately when symptoms and signs suspicious of SARS manifested clinically, strict infection control was practiced, and no secondary transmission of the SARS virus within the cohort was observed. This technique may ease the logistical constraints imposed by demands for large numbers of isolation facilities in the face of a massive outbreak.⁵⁵

Ancillary care centers. The expansion of the health-care system into additional community facilities can increase capacity for both inpatient and outpatient care, allowing hospital resources to be redirected to care for the most seriously ill. In 1998 the DoD's Biological Weapons Improved Response Program conducted a series of multiagency workshops on improved management of bioterrorism consequences, resulting in the Modular Emergency Medical System (Figure 19-4). The system is based on the rapid organization of a community's

medical assets into two types of expandable patient-care modules: (1) the acute care center (ACC) and (2) the neighborhood emergency help center.

ACC facilities, as well as associated medical personnel and supplies, will be most efficient if they are used for victims of bioterrorism-related illness only, because most patients will require similar treatment, and cohorting patients limits the exposure of noninfected persons. Patients who require acute or critical medical treatment of urgent conditions such as heart attacks, traumatic injuries, or severe exacerbations of chronic conditions should receive care at an existing medical facility with more diverse resources, regardless of their exposure status.

ACC planning should include several considerations: (a) the use of either temporary or fixed facilities; (b) location of the facilities; (c) availability of parking and ease of access; and (d) building conditions such as total space, layout, size of doorways and corridors, electrical supply, heating and air conditioning, lighting, floor coverings, hand-washing facilities, and refrigeration capabilities. Buildings suitable for use as an ACC include National Guard armories, gymnasiums, schools, hotel conference rooms, health clubs, and community centers, which usually contain separate rooms with large floor space for patient care. These buildings are

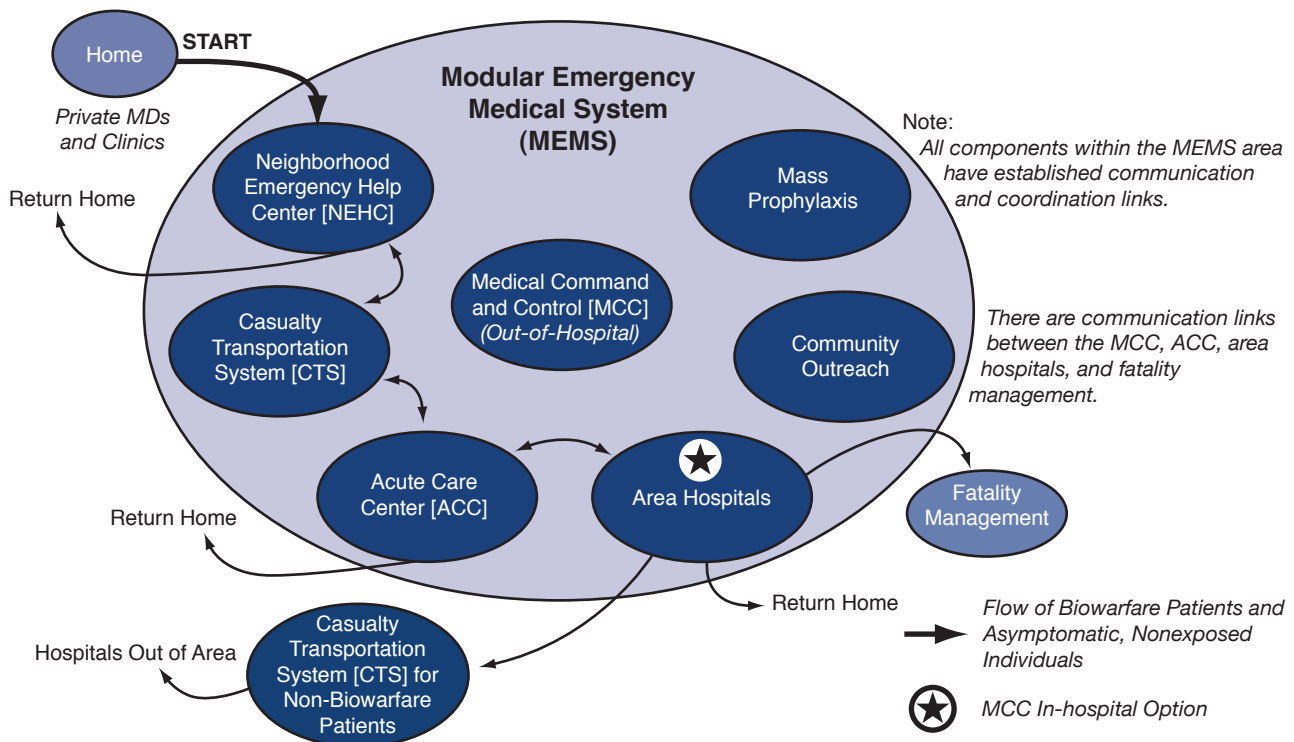


Fig. 19-4. Operational flow diagram of casualty evaluation and management using the Modular Emergency Medical System. Reproduced from: Acute Care Center: A Mass Casualty Care Strategy for Biological Terrorism Incidents. Aberdeen Proving Ground, Md: US Army Soldier and Biological Chemical Command; 2001.

likely to have bathrooms, kitchens, and laundry facilities with electrical and communication links, as well as adequate parking, loading ramps, and backup electrical generators. Schools and National Guard armories are generally publicly owned, which may make it easier for emergency officials to make use of them. Emergency planning officials should designate appropriate facilities in advance and begin to negotiate agreements for their use in mass casualty incidents.⁵⁶

All patients who receive treatment in an ACC facility should be accompanied by a functional medical record throughout their stay. Basic admission packages should consist of preprinted admission orders, medical history and physical checklists, multidisciplinary progress notes, and nursing flowsheets. Nursing documentation should be scaled down as much as possible, and charting by exception is highly recommended. To facilitate the transfer and management of patient information, ACCs should adopt the inpatient record system of the supporting hospital in the most simplified form possible.

ACC patients infected with contagious agents such as pneumonic plague or smallpox should not be discharged until they are deemed noninfectious. However, home-care instructions should be developed in case more people are exposed than can be admitted. Home-care instructions should provide information on the remaining treatment regime and any follow-up care that may be required. Patients should be discharged from the ACC when they can care for themselves (use the toilet and feed and dress themselves) or can stay with someone who can provide care.

An ACC site will only be successful if staffed by necessary medical and ancillary personnel. The suggested minimum staffing per 12-hour shift for a 50-bed nursing subunit is outlined in Exhibit 19-1. Staffing may be a problem because normally available personnel might not assist in a bioterrorism event, and if alternative sites are necessary, the normal healthcare system is running beyond capacity, stressing routine levels of staffing. The Rocky Mountain Regional Care Model for Bioterrorist Events has developed an alternative care site selection matrix tool⁴⁸ to help emergency medical planners judge the suitability of facilities, available at <http://www.ahrq.gov/research/altsites/>.

Mass Logistics

Local Stockpiles

The American Hospital Association's chemical and bioterrorism preparedness checklist recommends that each hospital have a 3-day supply of basic PPE (such as gloves, gowns, and shoe covers); a 3-day supply of specified pharmaceuticals; emergency power; a

loudspeaker or other mechanism to communicate with a large group of converging casualties outside the hospital entrance; and an external decontamination facility capable of handling 50 victims per hour. These guidelines give hospitals criteria by which they can measure their preparedness and improve their internal emergency response operation plans.⁵⁷ Hospital, state, and local officials have reported, however, that many hospitals needed additional equipment and capital improvements—including medical stockpiles, PPE, decontamination facilities, quarantine and isolation facilities, and air-handling and air-filtering equipment—to enhance preparedness.⁵⁸

Effective planning for a biological incident includes not only acquiring materials and pharmaceuticals based on the population and risk, but also determining what resources are available in the community. Counting resources available in the local community or region allows medical planners to leverage assets for a more comprehensive response. When the inventory is compared with the requirements determined by credible biological scenarios, logistical shortfalls can be identified and rectified. The Emergency Preparedness Resource Inventory, a software tool that assembles a regional resource inventory, has been pilot tested in an eight-county region of Pennsylvania. The inventory categorizes resources by type and location, including antibiotics, antidotes and antitoxins, beds, blood products, communications capability, communications, equipment, emergency response capability, emergency response equipment, EMS personnel, emer-

EXHIBIT 19-1

SUGGESTED MINIMUM STAFFING PER 12-HOUR SHIFT FOR A 50-BED NURSING SUBUNIT

- One physician
- One physician's assistant or nurse practitioner
- Six registered nurses or a mix of registered nurses and licensed practical nurses
- Four nursing assistants or nursing support technicians
- Two medical clerks (unit secretaries)
- One respiratory therapist
- One case manager
- One social worker
- Two housekeepers
- Two patient transporters

gency transportation equipment, facility size, facility and utility capabilities, intravenous fluids, licensed practical nurses, major medical equipment, medical supplies, medical therapists, nonmedical personnel, pharmacists, physicians, registered nurses, technicians, transportation, and vaccines.⁵⁹

Healthcare organizations may decide to develop local caches of medical supplies, including medications, vaccines, and patient care equipment. The Rocky Mountain Regional Care Model for Bioterrorist Events has adapted comprehensive lists of equipment and consumables developed by the Soldier's Biological and Chemical Command into three levels of medical caches for local hospitals (see Exhibit 19-2).

The Strategic National Stockpile

Local and regional stockpiles of medical supplies, equipment, and medications will likely not be able to meet the demand during a large-scale biological event. The SNS, managed by the CDC, is a national stockpile of medications, medical equipment, and supplies for use in the event of a terrorism event with chemical, biological, or radiological weapons. The

SNS was originally called the National Pharmaceutical Stockpile, which was created in 1999; it was renamed and moved to the DHS with the Homeland Security Act of 2002.

The SNS consists of massive stockpiles of pharmaceuticals, vaccines, medical supplies, equipment, and other items to augment local supplies of critical medical supplies. The program response is tiered; the initial shipment consists of over 100 cargo containers of 12-hour "push packages," which contain over 100 different product lines. Storage and staging facilities are located throughout the country, so that the push packages can reach the area of need within 12 hours. The shipment can arrive on a wide-body aircraft or seven tractor-trailers. Additional support can be provided through a vendor-managed inventory, which can be tailored to the size of the event and the agent involved. SNS resources are designed for mass patient care and prophylaxis; for example, the stockpile can provide 60-day prophylaxis against anthrax for 12 million people and treat more than 1.1 million symptomatic patients.⁶⁰

Although the SNS program deploys a technical advisory response unit to provide expert consultation

EXHIBIT 19-2

MEDICAL CACHE LEVELS FOR LOCAL HOSPITALS

Level I: Hospital Augmentation Cache

This cache consists of supplies for an increased surge capacity of 50 patients, including only items that have an extended shelf life: cots, linens, masks, gowns, gloves, intravenous injection poles, etc. This cache does not include any pharmaceuticals. Material in this cache may be packed in a trailer for mobility. The cache could be used as additional stock for an existing hospital (eg, to set up a medical ward in a cafeteria) or could provide supplies for limited-level care at an alternative site. If 11 hospitals acquired a cache, the total could provide the basic supply for a surge capacity of 550 patients in a metropolitan area of 1,000,000 people. Estimated cost for this cache (including trailer) is approximately \$20,000.

Level II: Regional Alternative Site Cache

This cache represents a more complete list of materials to supply a regional alternative care site for 500 patients. The materials in this cache could be packaged in a modular fashion, so that material to support multiples of 50 or 100 beds could be easily extracted. Approximate price for a single cache is currently less than \$100,000. As with the level I cache, only items with a long shelf life are included, and pharmaceuticals are excluded (it is assumed that the Strategic National Stockpile would provide pharmaceuticals within 72 hours of an event to augment levels I and II caches).

Level III: Comprehensive Alternative Care Site Cache

This cache, designed for a completely supplied 50-bed alternative care site, consists of items with both long and short shelf lives, including equipment, consumables related to patient care, administrative consumables, and oxygen and respiratory equipment. Material has been categorized, when possible, into use for quarantine and for caring for infectious and noninfectious patients.

Data source: Rocky Mountain Regional Care Model for Bioterrorist Events. Available at <http://www.ahrq.gov/research/altsites/alt-tool2.htm>. Accessed March 2, 2007.

on storage, repackaging, and distribution of supplies, state and local authorities are responsible for receipt, storage, and distribution. A coordinated plan for these logistics is the key to successful use of the SNS. Such a plan would include identifying the location to receive the stockpile (airfields with runways that can accept wide-bodied commercial jets) and ensuring that appropriate equipment is available to off-load the push packages. Supplies should be stored in a secured site with controlled temperature and humidity and access to highways and other transportation means.⁶⁰ Additionally, certain items may need to be broken down and repackaged before distribution.

Mass Prophylaxis

Whereas medications, medical supplies, and medical equipment required by hospitals may be transported directly to a healthcare facility, prophylactic medications or vaccinations may need to be distributed in large numbers to the community. In response to the anthrax mailings in 2001, representatives from the CDC, the US Public Health Service, and five disaster medical assistance teams were assembled within 18 hours. This group screened and offered postexposure prophylaxis to 7,076 postal workers over a 68-hour period.⁶¹ Aerosol dissemination of a biological agent would significantly increase the population at risk and the number of persons requiring prophylaxis or treatment, demanding significant coordination and personnel.

Uncommon antibiotics, antivirals, immunoglobulins, or vaccines may be necessary in certain situations. Hospitals, especially emergency departments, will be critical in administering prophylaxis to victims, staff, and members of the public, and must have ready access to large quantities of pharmaceuticals and supplies.⁶² Public fears may lead to a high demand for antibiotic prophylaxis during bioterrorism events; for example, during the 2001 anthrax mailings, a majority of emergency physicians encountered patients who requested anthrax prophylaxis. Strategies should be pursued to control inappropriate antibiotic allocation during bioterrorism events and ease the burden on front-line clinicians.⁶³

Every public health jurisdiction in the country is responsible for developing and maintaining the capability to respond to bioterrorism events, dispense antibiotics, and carry out vaccination campaigns tailored to its local population. Local response is necessary because mass prophylaxis activities must be operational before the arrival of state or federal resources; federal or state responders will likely require assistance from the community; a mass prophylaxis operation may remain under local control even after state and federal

assets arrive; and follow-up operations may continue after their departure.⁶⁴

Prophylaxis can be distributed to the community through a “push” or a “pull” system. In a push system, pharmaceuticals are brought to the individual; for example, the US Postal Service could deliver prepackaged medications to households. However, in this system, doses could not be modified based on weight, age, and comorbid conditions, nor could contraindications be evaluated. A pull system requires community members to come to a designated center to be evaluated and receive prophylaxis. The principal operating unit of this system is the point of distribution (POD).

Establishment of a POD requires detailed planning and preevent exercises incorporating local healthcare organizations, public health officials, law enforcement, the media, and emergency management planners. The plan needs to outline how the POD will function, how it will be staffed, what its operational protocols and procedures will be, and how it will be supplied. POD locations should have adequate storage capacity, ease of access, a communications system, and security; and POD activation plans should include triggers at the local, regional, and federal level.

Common POD operational concepts are depicted in Figures 19-5 and 19-6. Features include an initial greeting to direct the flow of patients at the entrance, distribution of demographic forms, and triage to identify those who are symptomatic, those who have definitely been exposed, those who may have been exposed, and those who have definitely not been exposed. The greeter identifies those who are not feeling well or believe they have been exposed to the biological agent or a contagious person. Those who are significantly ill are transported to a medical care facility. Those who are contacts or have been exposed may be moved to an isolation or quarantine facility, especially if they decline available prophylaxis or vaccination. A screening medical evaluation should be performed as well as a mental health evaluation if needed. A briefing on the agent released, the signs and symptoms of disease, the capacity to transmit the disease within the community, and the recommended treatment should be given. Finally, an evaluation for prophylactic medications or vaccination should be made, the medication or vaccination should be administered, and all forms should be collected.

The Bioterrorism and Epidemic Response Model, created by researchers at Weill Medical College of Cornell University in 2003 under contract to DHHS, is available to help determine requirements for community prophylaxis. Inputs into the model include the scale of the event; the size of the population; the extent

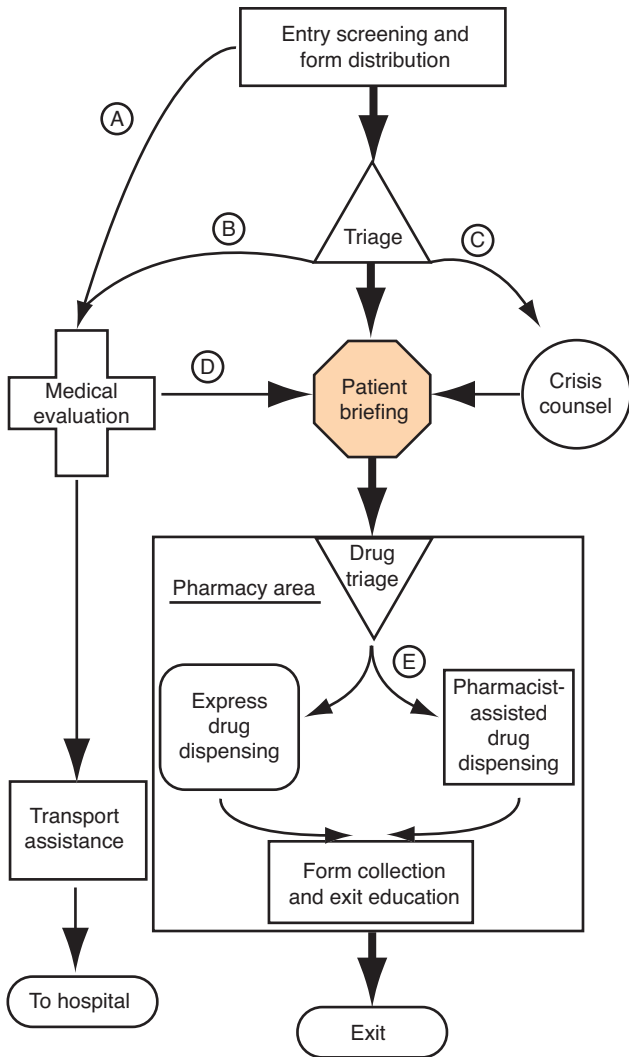
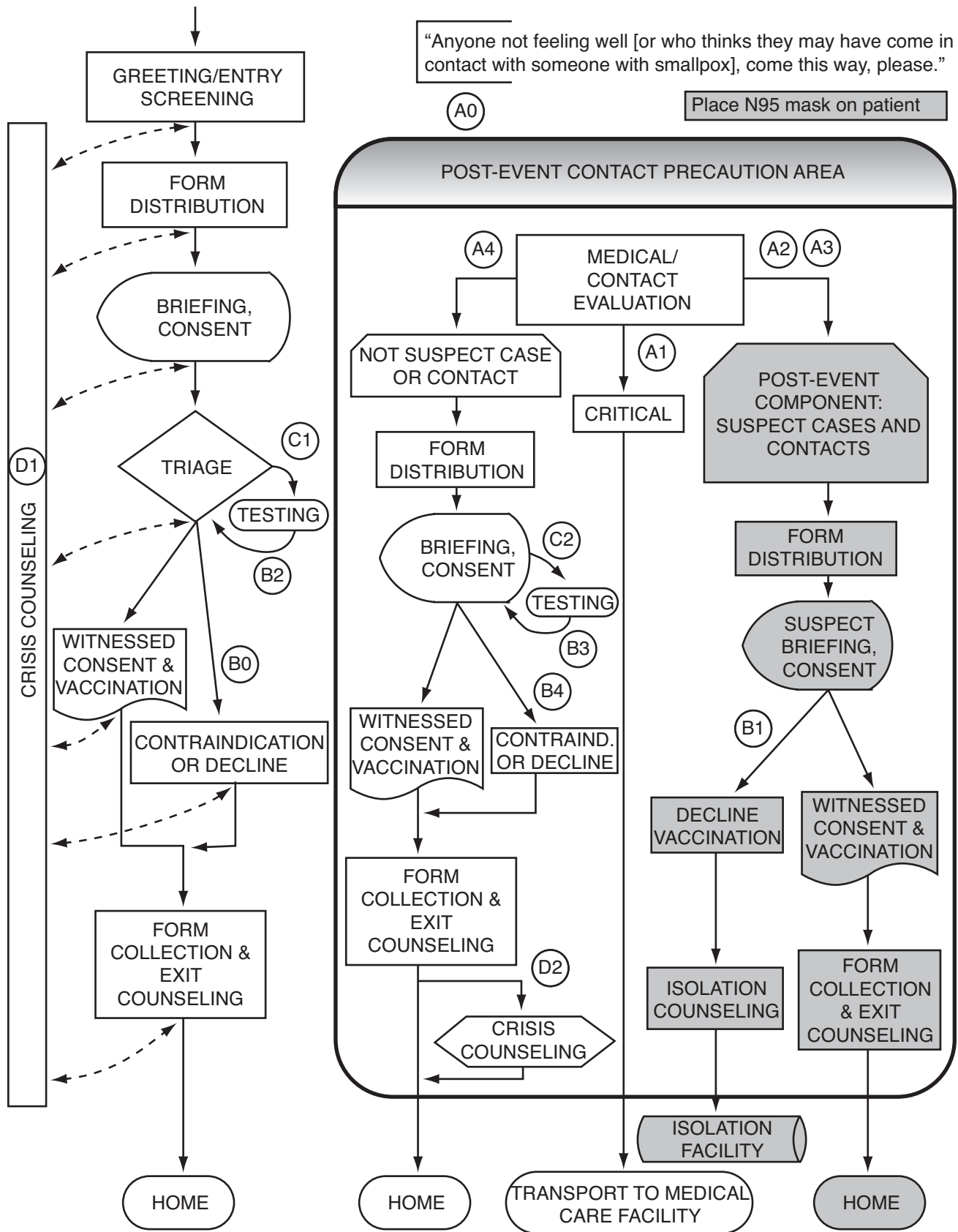


Fig. 19-5. Prophylaxis distribution center flow diagram. This process would be used in response to a mass exposure to anthrax or another noncommunicable agent, requiring the distribution of antibiotics. Patients arrive and are screened for visible signs of illness; those who are ill are sent to medical evaluation (a). The remaining patients are given any necessary forms and undergo triage, at which time they are sent to medical evaluation (b), mental health/crisis counseling (c), or patient briefings (d). A certain portion of patients who undergo medical evaluation come back through the briefings as well. Patients who are seriously ill are transported to hospitals or other medical care facilities. Those who finish briefings are sent to the drug triage area (e), where appropriate decisions are made regarding dispensing antibiotics or vaccination. Those with uncomplicated cases may go to express drug dispensing tables, whereas those with complicated cases may require assistance from pharmacists or other health professionals. Before patients leave, all forms and paperwork are collected in designated areas.

of the disease's transmission in the community; the duration of the campaign; the hours of POD operation and its downtime; the number of shifts worked in the POD; and whether crisis counseling and testing are to be offered in the POD. The model will then give the estimated patient flow rate of the POD, the number of PODs needed, the total number of staff required, the specific staffing requirements at each station, and the support staff required.⁶⁵ This tool is available at <http://www.ahrq.gov/research/biomodel.htm>. Additionally, the CDC offers free planning software for large-scale smallpox prophylaxis clinics that may be

Fig. 19-6. Flow diagram for a campaign responding to smallpox or other communicable agent. Patients are greeted at the front door of this clinic by screeners who ask if anyone is symptomatic or had contact with an infected individual. Those who are symptomatic or are suspected contacts are sent to a contact precaution area that is separate from the main area of activity in order to minimize the risk of contagion. Patients in the main (non contact precaution area) are given necessary forms and undergo briefings and triage. Clinics may offer testing including pregnancy and/or rapid HIV testing depending on the event, response, and availability of supplies and staff to perform tests. Written consents and vaccinations may need to be witnessed. Clinics may offer crisis/mental health counseling on site. Prior to exit, patients receive counseling on vaccination site care and follow-up and turn in forms. Patients in the contact precaution area are immediately taken to medical evaluation at which point they are classified as seriously ill requiring transfer to a hospital or other medical care facility, a suspect case or contact, or not a suspect case or contact. Patients in the latter two categories are then given necessary forms, briefings, triage, testing, vaccination, or other dispensing, and exit counseling much like patients outside the contact precaution area. One major difference is that suspected cases or contacts who refuse prophylactic medications or vaccination may be placed in isolation depending on the setting and applicable public health regulations. The diagram includes special isolation counseling for these individuals. Reproduced from: Hupert N, Cuomo J, Callahan MA, Mushlin AI, Morse SS. *Community-Based Mass Prophylaxis: A Planning Guide for Public Health Preparedness*. Rockville, Md: Agency for Healthcare Research and Quality; August 2004. AHRQ Pub 04-0044. Rockville, MD. Available at: <http://www.ahrq.gov/research/cbmprophyl/cbmpro.htm>. Accessed March 1, 2007.



downloaded at <http://www.bt.cdc.gov/agent/smallpox/vaccination/maxi-vac/>.

Because of limited local pharmaceutical supplies, plans should include mobilization of regional resources and the SNS.⁶⁶ PODs may be initiated and staffed by active-duty military, National Guard units, US Public Health Service members, disaster medical assistance teams, or local medical personnel. Several public health centers are using similar models for distributing annual influenza vaccinations as an exercise for possible outbreaks of biological agent attacks. Some health departments have even created “drive through” vaccine distribution centers.⁶⁷

Mass Fatalities

Response to a biological event includes mortuary affairs and the disposition of infected remains. Healthcare organizations must have well-formed procedures for handling, storing, and managing large numbers of contaminated human remains, developed in coordination with local medical examiners and coroners based on available assets. Medical examiners and coroners must coordinate activities with several agencies including law enforcement, design a geographic strategy to manage mortuary affairs operations, mitigate the contamination from human remains and maintain biosafety considerations, and manage a personal effects depot.

Code of Federal Regulations 49⁶⁸ governs the transport of infectious substances and requires the substances to be labeled and packaged appropriately. Section 173.196 states that infected human remains are considered infectious substances that must be packaged to standard, including the use of one or more inner leak-proof packages and an outer package with material sufficient to absorb the entire contents of the inner package. The entire package must be strong and secured against movement, and it must not be reopened after arriving at its destination.

The capability of cemeteries to bury the number of remains from an event must be considered in planning. Additionally, many agencies have yet to confirm environmental hazards associated with burial of large numbers of contaminated remains, and cemetery owners may require authorities to provide indemnity from

future citation.⁶⁹ Cremation is the disposition of choice for highly infectious remains, but may not always be practical. As a rule, cremation takes approximately 3 hours per body, which may be impractical if there are large numbers of fatalities. Additionally, crematoriums must have a retort system that captures and burns particles in the smoke before it is released into the atmosphere.

Current procedures for handling remains of patients who succumbed to infectious diseases are based on mode of transmission of the disease. For example, the recommendation for handling corpses with *Mycobacterium tuberculosis* includes using NIOSH-approved HEPA filters, temporarily placing a surgical mask or disposable cloth over the body’s mouth and nose to prevent possible generation of any aerosols, placing the remains and disassociated portions in plastic burial pouches, and using negative-pressure rooms that provide at least 12 air exchanges per hour. Electric saws should be equipped with protective guards and vacuum attachments to capture and remove aerosolized contaminants.⁷⁰

Recommendations for handling remains of anthrax victims include the use of standard precautions during general handling, with additional respiratory PPE when performing activities that generate aerosols. Autopsies should be performed with respiratory PPE, and under BSL-3 conditions during activities with a potential to create aerosols. During burial, contact with corpses should be limited to personnel wearing PPE, embalming should be avoided, and the body should be packaged in leak-proof containers and buried without reopening the casket.

The recommendations for remains of patients dying from plague are similar to those of anthrax fatalities. For smallpox-contaminated remains, the same general recommendations as for anthrax and plague apply; additionally, only personnel who have received the smallpox vaccine, or who will be subsequently placed on fever watch, should handle the remains, and autopsies should be performed only if absolutely needed. Corpses of patients who had viral hemorrhagic fevers should be autopsied only in BSL-4 conditions, with the use of additional respiratory PPE during handling.

LEGAL ISSUES

Legal counsel should be included in all steps of disaster preparedness and response planning to assist with ensuring adequate building and health codes, enforcing quarantine, and protecting medical workers and volunteers from liability. Occupational Safety and Health Administration regulations on PPE should be followed.

The standard of medical care may need to be altered in a large-scale response. Although the term “altered standards” has not been clearly defined, it is generally assumed to mean “a shift to providing care and allocating scarce equipment, supplies, and personnel in a way that saves the largest number of lives in con-

trast to the traditional focus on saving individuals.” Examples of altered standards of care in response to a biological incident include changing infection control standards to permit group-isolation rather than single-person isolation units; creating alternate care sites in facilities not designed to provide medical care, such as schools, churches, or hotels; changing the personnel who provide various kinds of care; and temporarily changing privacy and confidentiality protections. It is important to establish clear authority to activate the use of altered standards of health and medical care. Minimally accepted levels of care documentation provided to an individual may have to be established, both for patient care quality and as the basis for reimbursement from third-party payers.⁷¹ Additionally, healthcare providers may be asked to perform above their training or credentialing. During the 1918–1919 influenza outbreak, states used dentists as physicians, graduated medical students early, and expedited medical board examinations to provide more physicians.⁴¹

Declaration of quarantine, not just for those who are ill but also for their contacts and contacts of contacts, can have legal implications, especially when dealing with those who refuse quarantine. Key factors in quarantine compliance in Canada during the SARS outbreak included fears of income loss, consistent information about the threat and measures to contain it, and adequate logistical and psychological support to those quarantined.⁷² Each of these factors should be addressed in a quarantine plan.

Prohibiting direct military involvement in law enforcement is in keeping with long-standing US law and policy limiting the military’s role in domestic affairs. The Posse Comitatus Act was enacted after the Civil War in response to the perceived misuse of federal troops who were charged with domestic law enforcement in the South.⁷³ It has come to symbolize the separation of civilian affairs from military influence. The act generally prohibits US military personnel from interdicting vehicles, vessels, and aircraft; conducting surveillance, searches, pursuit, and seizures; or making arrests on behalf of civilian law enforcement authorities. The act states:

Whoever, except in cases and under circumstances expressly authorized by the Constitution or Act of Congress, willfully uses any part of the Army or Air Force as a posse comitatus or otherwise to execute the laws shall be fined under this title or imprisoned not more than two years, or both.⁷³

DoD Directive 5525.5⁷⁴ extended the act’s substantive prohibitions to the US Navy and Marine Corps. The act does not apply to the US Coast Guard in peacetime or to the National Guard when not in federal service. However, Congress has enacted exceptions to the law that allow the military to assist civilian law enforcement agencies in certain situations, most commonly in illegal drug enforcement. Other examples include the following:

- The Insurrection Act,⁷⁵ which allows the president to use military personnel at the request of the state legislature or governor to suppress insurrections. It also allows the president to use federal troops to enforce federal laws when rebellion against US authority makes it impracticable to use traditional law enforcement authorities.
- Title 18 United State Code Section 831⁷⁶ permits DoD personnel to assist the Justice Department in enforcing prohibitions regarding nuclear materials, when the attorney general and the secretary of defense jointly determine that an “emergency situation” exists that poses a serious threat to US interests and is beyond the capability of civilian law enforcement agencies.
- When the attorney general and the secretary of defense jointly determine that an “emergency situation” exists that poses a serious threat to US interests and is beyond the capability of civilian law enforcement agencies. DoD personnel may assist the Justice Department in enforcing prohibitions regarding biological or chemical weapons of mass destruction.⁷⁷

SUMMARY

Response to a bioterrorism event or outbreak of emerging diseases can rapidly overwhelm the country’s current medical infrastructure. To respond appropriately, officials must create response plans and provide the necessary resources to mitigate these events. The economic implications of preparedness are substantial. A 1997 model predicted that the economic impact of a bioterrorism attack could range from \$477.7 million to \$26.2 billion per 100,000 persons exposed,

depending on the agent.⁷⁸ Planning, streamlined surge capacity, and initiation of early prophylaxis may drastically decrease these figures. Healthcare organizations must know their capabilities for patient care and logistics and have plans in place to leverage assets at the local, regional, state, and federal level to provide an adequate response. Military medical units must be prepared to respond to incidents at their locations as well as work with the civilian community.

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Chapter 20

MEDICAL MANAGEMENT OF POTENTIAL BIOLOGICAL CASUALTIES: A STEPWISE APPROACH

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INTRODUCTION

A 10-STEP APPROACH TO CASUALTY MANAGEMENT

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Step 2: Protect Yourself

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Step 4: Disinfect or Decontaminate as Appropriate

Step 5: Establish a Diagnosis (the 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

If the identity of an agent used in a biological attack is known, response to such an attack is, in some sense, relatively straightforward. Earlier chapters in this volume deal with diagnoses and treatment strategies specific to known infectious and toxic agents. A larger problem arises, however, when the identity of an agent is uncertain. In some cases, a biological attack might be threatened or suspected, but it may remain unclear if such an attack has actually occurred. Moreover, it may be unclear whether casualties in certain situations arise from exposure to a biological, chemical, or radiological agent; result from a naturally occurring infectious disease process or toxic industrial exposure; or simply reflect a heightened awareness of background disease

within a community or population. Recent experiences with West Nile virus,¹ severe acute respiratory syndrome,² pneumonic tularemia,^{3,4} and monkeypox⁵ highlight this dilemma. In each case, the possibility of bioterrorism was raised, although each outbreak was ultimately proven to have had an innocuous origin. In some instances, proof of such origins can be difficult or impossible to obtain, thus providing plausible deniability—or the precise reason some bioterrorists choose specific 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 population.

A 10-STEP APPROACH TO CASUALTY MANAGEMENT

In responding to the unknown, it is helpful, in many situations, to use a standardized, stepwise approach. This would be especially true with a medical mass casualty event, in which the use of such an approach (as advocated by the Advanced Trauma Life Support model sponsored by the American College of Surgeons⁶) is already well accepted and practiced. This stepwise approach would also be helpful under austere or battlefield conditions. Although major theater-level (level 4) and continental United States-based (level 5) military medical centers (and research institutions such as the US Army Medical Research Institute of Infectious Diseases [USAMRIID]) and the US Army Medical Research Institute of Chemical Defense may possess sophisticated diagnostic and response capabilities, the medical provider on the battlefield and at lower level medical treatment facilities is typically required to make rapid, therapeutic decisions based on incomplete information and with little immediate support. Civilian clinicians, first responders, and public health personnel who practice 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 medical mass casualty trauma event, these decisions may have life-and-death implications. In these situations, a stepwise or algorithmic approach becomes invaluable.

USAMRIID has developed a 10-step approach to the management of casualties that might result from biological warfare or terrorism:

1. Maintain a healthy index of suspicion.
2. Protect yourself.
3. Save the patient's life (the primary assessment).
4. Disinfect or decontaminate as appropriate.
5. Establish a diagnosis (the secondary assessment).

6. Provide prompt therapy.
7. Institute proper infection control measures.
8. Alert the proper authorities.
9. Conduct an epidemiological investigation and manage the psychological aftermath of a biological attack.
10. Maintain a level of proficiency.

Many facets of this approach could also be helpful in dealing with potential chemical or radiological casualties. It is no longer adequate for clinicians and medical personnel simply to understand disease processes. Rather, these personnel (whether military or civilian) must have tactical, operational, and strategic knowledge of threat response (and knowledge 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 have traditionally been the realm of the individual clinician.
- Operational response involves the mechanisms by which the provider interacts with his or her institution (eg, hospital, clinic, medical unit) to provide mass care during a disaster.
- Strategic response involves systemwide disaster preparedness and response. In a civilian setting, the response would include mechanisms by which state and federal disaster response elements might become involved.

Currently, medical personnel 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.

In the 10-step USAMRIID approach, steps 1 to 7 deal predominately with tactical response (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 the system as a whole). Derivation of this 10-step approach is reported elsewhere,⁷⁻¹⁰ and a condensed version of it appears in recent editions of USAMRIID's *Medical Management of Biological Casualties Handbook* (or the *Blue Book*).¹¹ The following is an overview of this stepwise approach.

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. Most likely, victims would be tightly clustered in time and space (ie, they would succumb in close proximity—both temporally and geographically—to a dispersal device). Complicating discovery of the intentional nature of a biological attack, however, is the fact that biological agents possess inherent incubation periods, whereas conventional, chemical, and nuclear weapons do not. These incubation periods, typically lasting several days (but up to several weeks as with *Coxiella burnetii* and *Brucellae*), allow for the wide dispersion of victims in time and space. Additionally, incubation periods make it likely that the first responders to a biological attack would not be firemen, policemen, paramedics, or other traditional first responders, but rather primary care providers, hospital emergency departments, and public health officials. In these circumstances, maintenance of a healthy index of suspicion is imperative.

In some instances, maintaining an index of suspicion might be easy because patients with diseases caused by biological agents may present with specific characteristic clinical findings, which result in a very limited differential diagnosis. The hallmark presentation of inhalational anthrax is a widened mediastinum, a clinical finding seen in few naturally occurring conditions. In botulism, the hallmark presentation is a descending, symmetric, flaccid paralysis. Whereas an individual patient with flaccid paralysis might prompt consideration of disorders such as Guillain-Barre syndrome, Eaton-Lambert syndrome, poliomyelitis, and myasthenia gravis, the near-simultaneous presentation of multiple patients with flaccid paralysis should quickly prompt consideration of a diagnosis of botulism. Similarly, persons with plague often exhibit hemoptysis in the later stages of illness. Such a finding is uncommon among previously healthy individuals, but it can be caused by tuberculosis, staphylococcal and *Klebsiella pneumoniae*, carcinoma, and trauma. Multiple patients with hemoptysis, however, should

prompt consideration of a diagnosis of plague. Smallpox is characterized by a unique exanthem, perhaps like *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 likely to be ineffective. Therefore, therapy is best instituted during the incubation or prodromal phases of these diseases if it is to be beneficial. Unfortunately, however, in their prodromal forms these diseases are likely to appear as simple, undifferentiated febrile illnesses, difficult (if not impossible) to distinguish from other common infectious diseases. Similarly, many other diseases potentially arising from a biological attack (eg, tularemia, brucellosis, melioidosis, Q fever, staphylococcal enterotoxin intoxication, and Venezuelan equine encephalitis) appear simply as undifferentiated febrile illnesses throughout their disease course. Prompt diagnosis and institution of targeted therapy are possible only with the maintenance of a very high index of suspicion.

Epidemiological clues can lead the clinician to suspect that a disease outbreak may have been intentional.¹² Large numbers of persons 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 warrants further investigation. Simultaneous outbreaks of a disease in noncontiguous areas should prompt consideration of an intentional release, as should simultaneous or sequential outbreaks of different diseases in the same locale. Even single cases of uncommon illness, such as anthrax or certain viral hemorrhagic fevers (Ebola, Marburg, Lassa, etc), would be suspicious, and a single case of smallpox 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 unintentional aerosol release. Evidence of a disparate attack rate between individuals known to be indoors and outdoors at a given time should also be sought out and evaluated. Intelligence reports, terrorist claims, and the discovery of aerosol spray devices would lend credence to the theory that a disease outbreak was of sinister origin. The epidemiological clues to a bioterrorist attack are summarized in Exhibit 20-1.

On the modern battlefield, an array of developing technology is increasingly 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 Biological Warfare Agent

EXHIBIT 20-1

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;5:528–530.

Detection System (Bio-Rad Laboratories, Hercules, Calif) is the first automated biological detection system of the US Department of Defense. It was designed to provide fixed-site protection to air and port facilities. The Portal Shield is equipped with modular sensors capable of simultaneously assaying for eight different threat agents and providing presumptive identification within about 25 minutes. The Biological Integrated Detection System (BIDS; Battelle, Columbus, Ohio) is a high-mobility, multipurpose, wheeled, vehicle-mounted system (Figure 20-1) equipped with samplers, an aerodynamic particle sizer, a flow cytometer, a chemical-biological mass spectrometer, and other sophisticated assays to permit rapid, real-time detection of multiple biological threat agents on the battlefield. BIDS was first fielded as a single company of 38 units in 1996; current plans call for a dramatic expansion of BIDS capabilities, with 17 companies planned by the end of 2009. The Joint Biological Point Detection System (Battelle, Columbus, Ohio) is the next-generation successor to the BIDS and is envisioned as integrating into the BIDS platform. Purportedly, the Joint Biological Point Detection System will be capable of definitively identifying biowarfare threat agents within 15 minutes. Until such technology is refined, validated,

and made widely available, clinicians, health officials, chemical personnel, and commanders must rely on clinical, epidemiological, and intelligence clues to maintain their index of suspicion.

Step 2: Protect Yourself

Providers who become casualties themselves are of little use to their patients. Before approaching casualties of biological or chemical warfare or victims of a terrorist attack, clinicians should be familiar with the basic means of self-protection. Generally, these protective measures fall into one of three categories: (1) physical protection, (2) chemical protection, and (3) immunological protection. Under a given set of circumstances, clinicians 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-filled chemical protective overgarments. Although military-style protective clothing and masks were designed with chemical agent protection in mind, they are also capable of offering protection against biological agents. Even though some 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 probably be unavailable to civilians at the precise moment a threatening agent is released. The unannounced release of colorless and odorless biological agents by terrorists



Fig. 20-1. 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.

would afford people no opportunity to don such protective gear, even if it was available. The misuse of protective equipment in the past has led to fatalities, including the suffocation of infants and adults in protective ensembles.^{13,14} Although military chemical-biological masks—such as the M40/42 series (ILC Dover LP, Frederica, Del), the M45 series (ILC Dover LP, Frederica, Del), the M43/48 series (for aviators; ILC Dover LP, Frederica, Del), and the next-generation XM50 series (known as the JSGPM or the Joint Service General Purpose Mask; Avon Rubber plc, Melksham, Wiltshire, UK)—provide ample protection against inhalation hazards posed by chemical and biological weapons, as well as radioactive dust particles, they are potentially mission degrading and are unnecessary if and when the threat is limited to biological agents. A simple surgical mask will protect against inhalation of infectious aerosols of virtually any of the biological agents typically described in a terrorism context. The lone 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, agents in the Centers for Disease Control and Prevention (CDC) categories A and B (Table 20-1) are not contagious by the respiratory route. Thus, respiratory tract protection is 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/Storm, tens of thousands of US troops were given pyridostigmine under an emergency use authorization. In early 2003 the US Food and Drug Administration (FDA) gave its final approval for use of pyridostigmine bromide as a “preexposure” means of prophylaxis against intoxication with soman, an organophosphate-based chemical nerve agent. Similar strategies might be used against biological weapons. For example, if a specific terrorist group possessing a specific weaponized agent was known to be operating in a given locale, public health authorities might contemplate the widespread distribution of an appropriate prophylactic antibiotic. Opportunities to implement such a strategy, however, remain limited.

Immunological Protection

For the near future, active immunization may offer one of the most practical methods for providing pre-exposure prophylaxis against biological attack. In the military, decisions about vaccination are made at the highest levels of policy making, typically 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 particular circumstance is a

**TABLE 20-1
CRITICAL AGENTS FOR HEALTH PREPAREDNESS**

Category A*	Category B†	Category C‡
Variola virus	<i>Coxiella burnetii</i>	Nipah virus
<i>Bacillus anthracis</i>	<i>Brucellae</i>	Hantaviruses
<i>Yersinia pestis</i>	<i>Burkholderia mallei</i>	Yellow fever virus
Botulinum toxin	<i>Burkholderia pseudomallei</i>	Drug-resistant tuberculosis
<i>Francisella tularensis</i>	Alphaviruses	Tick-borne encephalitis
Filoviruses and arenaviruses	Certain toxins (ricin, staphylococcal enterotoxin B, trichothecenes)	
	Food safety threat agents (<i>Salmonellae</i> , <i>Escherichia coli</i> O157:H7)	
	Water safety threat agents (<i>Vibrio cholerae</i>)	

*Agents with high public health impact requiring intensive public health preparedness and intervention.

†Agents with a lesser need for public health preparedness.

‡Other biological agents that may emerge as future threats to public health.

Adapted from Centers for Disease Control and Prevention. Biological and chemical terrorism: strategic plan for preparedness and response. Recommendations of the CDC Strategic Planning Workgroup. *MMWR Recomm Rep.* 2000;49(RR-4):1-14.

complex one that must include a careful risk–benefit calculation. During Operations Desert Shield / Storm, about 150,000 service members received at least one dose of anthrax vaccine, and about 8,000 service members received botulinum toxoid. Since 1998 the US military has intermittently used force-wide anthrax vaccination, and since 2003 the US military has administered smallpox vaccine to deploying troops and certain medical response teams.

In a civilian counterterrorism context, the decision to use a specific vaccine is perhaps even more complex. Factors that would influence a decision by public health officials to recommend vaccination include the following:

- Intelligence
 - How likely and/or plausible is an attack?
 - How imminent is the threat?
 - How specific is the threat?
- Vaccine safety
- Vaccine availability
- Disease consequences
 - Is the threat from a lethal agent?
 - Is the threat from an incapacitant?
- Availability of postexposure prophylaxis and/or therapy

Recently, civilian public health and policy planners have considered the widespread distribution of anthrax and smallpox vaccines.

Anthrax. Anthrax vaccine adsorbed (AVA [BioThrax]; BioPort Corporation, Lansing, Mich) 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, this vaccine likely is also effective in preventing inhalational anthrax.¹⁶ At least 20 clinical studies, surveys, and reports demonstrate the safety of AVA,^{17,18} and the FDA recently reaffirmed the vaccine as safe and effective.¹⁹ Whereas widespread usage of AVA has occurred within the US military (as of September 2005, more than 5.2 million doses of AVA had been given to more than 1.3 million service members), logistical and other considerations make large-scale civilian employment impractical at present. The vaccine is licensed as a six-dose series, given at 0, 2, and 4 weeks, and at 6, 12, and 18 months. Yearly boosters are recommended for those with ongoing risk

of exposure. The FDA approves AVA only for persons between the ages of 18 to 65, further complicating any potential civilian anthrax vaccination strategy. Although a large-scale preexposure offering of AVA to the general public might be problematic, some experts recommend that three doses of the vaccine, given simultaneously with antibiotics, may enhance protection and/or enable the clinician to shorten a postexposure antibiotic course.²⁰ According to some experts, a three-dose series of AVA (given at time 0 and at 2 and 4 weeks after the initial dose)—combined with 30 days of antibiotics—might be an acceptable alternative to longer (60–100-day) antibiotic courses alone in the treatment of, or postexposure prophylaxis against, inhalational anthrax. Currently, no human studies 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. In December 2002, a plan to vaccinate selected US healthcare workers and military personnel was announced. Within the Department of Defense, service members deploying to locations believed at risk for biological attack and members of designated smallpox epidemiological and clinical response teams were selected for vaccination. As of September 30, 2005, 875,890 military response team members, hospital workers, and operational forces had been vaccinated, with one death that occurred from a lupus-like illness. Although the emergence of myopericarditis (there were 102 confirmed, suspected, or probable cases among the vaccinees) as a complication of vaccination²¹ led to a revision of prevaccine screening (candidates with multiple cardiac risk factors are now excluded), rates of other adverse reactions were low. No cases of eczema vaccinatum, fetal vaccinia, or progressive vaccinia occurred. Only 84 cases of autoinoculation and 54 instances of transfer of vaccinia to family members and other intimate contacts occurred.²² Vaccinia immune globulin was required on only three occasions: to treat two patients with ocular vaccinia²³ and to treat a burn patient with a recent immunization. The success of this program suggests that mass vaccination can be accomplished with greater safety than previously believed.²⁴

Whereas universal civilian vaccination was not recommended under the vaccination plan, the possibility of a future strategy calling for such recommendations arose, and provisions were made to provide smallpox vaccine to members of the general public who specifically requested it. The risk–benefit analysis of this widespread civilian vaccination is difficult to assess. Risks of smallpox vaccination are well known and can be significant.^{25,26} The benefits of a civilian vaccination program, however, are less well determined; although

the global eradication of smallpox is one of the greatest public health accomplishments—and the wisdom of administering vaccination with live vaccines remained unquestioned during the era of endemic smallpox—the likelihood of contracting smallpox today via a terrorist attack is unknown. Thus, the risk–benefit calculation in this scenario is not based solely on medical considerations, but also on intelligence estimates.

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 programs, 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 vaccine supply, safety, and logistics issues.^{27,28}

A large number of persons are at risk for severe vaccine reactions today, compared with the previous era of routine civilian smallpox vaccination, which ended in 1972. This increase in risk is due to an estimated 10 million persons in the United States who have compromised immunity associated with the human immunodeficiency virus, the 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.²⁹ 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 if the probability of a large-scale attack is high. Prerelease mass vaccination of healthcare workers, however, could be considered in the setting of lower attack probability because of the risk of exposure while caring for patients and the benefit of keeping healthcare workers healthy and functioning in an epidemic setting.³⁰

The smallpox vaccine used in the United States is Dryvax (Wyeth Laboratories, Marietta, Pa), a preparation derived from the harvested lymph of calves inoculated with a strain of vaccinia virus, an orthopoxvirus closely related to the variola virus. Production of Dryvax ceased in 1981, and lots in use are at least 25 years old. A new cell-culture derived vaccinia has been licensed by the FDA (September 2007); 300 million doses have been stockpiled by the US Department of Health and Human Services for emergency use. This vaccine is relatively easy to mass produce. These new

vaccines are produced in cell culture rather than in calf lymph. It is unlikely that this will significantly diminish the risk of adverse reactions, however, because the new vaccines will use the same live strain of vaccinia virus. The majority of adverse reactions to current vaccinia virus-containing vaccines are derived from the live nature of the virus rather than the method of preparation. To minimize the risks to immunocompromised vaccine recipients, the US Department of Health and Human Services awarded a contract to add 20 million doses of a highly attenuated smallpox vaccine, modified vaccinia Ankara, to the national biodefense stockpile. This vaccine is undergoing completion of phase II clinical trials in both healthy and immunocompromised subjects.

Release of civilian Dryvax stocks is controlled by the CDC, and conditions for such release have been established.³¹ The current CDC smallpox response strategy is based on preexposure vaccination of carefully screened first responders and members of epidemiological and clinical response teams. The CDC plans also provide for a program to treat certain severe complications of vaccination using vaccinia immune globulin under an investigational new drug protocol, as well as for compensation of people experiencing such complications through the Smallpox Vaccine Injury Compensation Program (US Department of Health and Human Services, Health Resources and Services Administration, Merrifield, Va).³²

The CDC 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 instances in which the number of cases or the location of cases renders the ring strategy inefficient, or if the risk of additional smallpox releases is high.³³ 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.³⁴ Controversy exists among experts regarding the predicted benefit of postrelease mass vaccination from the 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.^{35,36} Vaccination is one component of a multifaceted response, which should also include the following:

- 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. Implementation of any such strategy would be problematic. A vaccine against plague, previously licensed in the United States, is no longer produced. This vaccine, which required a three-dose primary series that was followed by annual boosters, was licensed only for persons 18 to 61 years old. Although reasonably effective against bubonic plague and widely used by the Department of Defense to protect against endemic disease, the vaccine 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 (yellow fever) is widely available, although its causative virus is not regarded as a significant weaponization threat by most policy makers and health officials. The US military administered yellow fever vaccine to large numbers of troops to guard against endemic disease rather than a bioweapons threat. Additionally, a vaccine against Q fever (Q-Vax; [*C burnetii* vaccine; CSL Limited, Victoria, Australia]) is licensed in Australia. Although this vaccine might be a useful addition to the military biodefense armamentarium, the self-limited nature of Q fever makes it unlikely that widespread use of the 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, equine encephalitides, staphylococcal enterotoxins, ricin, viral hemorrhagic fevers, and other potential agents of bioterrorism are in various stages of development.³⁷ Investigational vaccines against tularemia, botulism, equine encephalitides (especially Venezuelan equine encephalitis), staphylococcal enterotoxin B, Q fever, and other agents have been used under investigational new drug protocols to protect USAMRIID scientists who study these agents.

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

Once self-protective measures are implemented, the clinician can approach the medical mass casualty event scenario and begin assessing patients (also known as the Primary Survey according to Advanced Trauma Life Support guidelines). This initial assessment is brief and 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. At this point, attention should therefore be focused on maintaining a patent airway and providing for adequate breathing and circulation. The need for decontamination and for

the administration of antidotes for rapid-acting chemical agents (eg, 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 Airway—which should be examined for the presence of conventional injury, but should also be examined because exposure to certain chemical agents (eg, mustard, Lewisite, or phosgene) can damage the airway.
- B Breathing—many agents of biological (and chemical) terrorism may cause the patient to experience respiratory difficulty (eg, anthrax, plague, tularemia, botulism, Q fever, staphylococcal enterotoxins, ricin, cyanide, nerve agents, and phosgene).
- C Circulation—which may be compromised because of conventional or traumatic injuries sustained during a medical mass casualty event, but may also be involved in septic shock associated with plague and in circulatory collapse associated with viral hemorrhagic fevers.
- D Disability—specifically, neuromuscular disability; note that botulism and nerve agent exposures are likely to present with a preponderance of neuromuscular symptomatology.
- E Exposure—In a medical mass casualty event setting, this serves as a reminder to remove the victim's clothing to perform a more thorough secondary assessment. At this point, the need for decontamination and disinfection is considered.

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 that are performed by unit personnel (guided or assisted by specific, highly trained Chemical Corps decontamination companies). However, decontamination, in the classical sense, may not be necessary after a biological attack (the same is not always true after a chemical attack) because of the inherent incubation periods of biological agents. Although patients will not typically become symptomatic until several days after exposure, they are likely to have bathed and changed clothing several times before presenting for medical care, thus effectively accomplishing self-decontamination. Exceptions might include persons directly exposed to an observed attack or persons encountering a substance in a threatening

letter, when common sense might dictate topical disinfection. Even in these situations, bathing with soap and water and using conventional laundry measures would be adequate. Situations such as 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. In the past, significant psychological stress has been caused by unnecessary, costly, and resource-intensive attempts at decontamination.³⁸ Some of these attempts have involved forced disrobing and showering in public streets, under the prurient eye of media cameras. These problems may be avoided by measured responses to the following³⁹:

- announced threat (or presumed hoax),
- telephoned threat and/or the empty letter,
- suspicious package, and
- the delivery device.

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. Whereas human and environmental health protection concerns take precedence over law enforcement procedures, threat and hoax scenarios require early involvement of law enforcement personnel and a respect for the need to maintain an uncompromised crime scene. Typically, decontamination or disinfection is not necessary.

The Telephoned Threat and/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 and/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 only 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 legal and public health investigations.

The Suspicious Package

When a package is discovered and found to contain powder, liquid, or other physical material, response should be individualized. However, 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 be promptly notified.

People who have come into contact with the contents should remove clothing as soon as practical and seal these items in a plastic bag. Persons should then wash with soap and water⁴⁰ and, in most cases, may be sent home after receiving adequate instructions for follow-up and providing contact information. In most cases, 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 (eg, eyeglasses, jewelry) may be washed with soap and water or immersed in 0.5% hypochlorite (household bleach diluted 10-fold) if a foreign substance has come in contact with 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

- US Army's 22nd Chemical Battalion (also known as the Technical Escort Unit; Aberdeen Proving Ground, Md),
- US Marine Corps' Chemical-Biological Incident Response Force (Camp Lejeune, NC), or
- Federal Bureau of Investigation's Hazardous Materials Response Unit (Washington, DC).

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 described previously (the Chemical-Biological Incident Response Force has its own extensive decontamination capabilities). Decisions regarding institution of empirical postexposure prophylaxis pending determination of the nature of the threat and identification of the involved biological agents should 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 minimize the disruption and cost associated with large-scale decontamination, costly hazardous material unit involvement, and the broad institution of therapeutic interventions. These professionals can help avoid widespread public panic.

Step 5: Establish a Diagnosis (the Secondary Assessment)

Once decontamination has been considered and accomplished if warranted, the clinician may perform a more thorough and targeted assessment aimed at establishing a diagnosis (also known as the Secondary Survey according to Advanced Trauma Life Support guidelines). The thoroughness and accuracy used to establish this diagnosis will vary depending on the circumstances of the clinician. At robust levels of care (levels 4 and 5), the clinician may have access to infectious disease and microbiology professionals, as well as to sophisticated diagnostic assays. Under these circumstances, it may be possible to formulate a definitive microbiological diagnosis promptly. However, it is equally conceivable that a primary care provider practicing at lower levels of care (levels 1–3) or in more austere circumstances may need to intervene promptly based on limited information and without immediate access to subspecialty consultation. Even in these cases, however, reasonable care can be instituted based on a syndromic diagnosis. An “AMPLE” history may aid in establishing this diagnosis:

- 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 eaten; and
- E environment/events preceding incident.

A brief but focused physical examination, even one performed by relatively inexperienced practitioners, can reveal at a minimum whether a victim of a biological or chemical attack exhibits primarily respiratory, neuromuscular, or dermatologic signs, or suffers from an undifferentiated febrile illness. By placing patients into one of these broad syndromic categories, empiric therapy can be initiated (see step 6). This empiric therapy can be refined and tailored once more information becomes available.^{41,42}

When the situation permits, laboratory studies should be obtained to assist with later definitive diagnosis. Suggested laboratory studies, not all of which

will be applicable in every case, are listed in Exhibit 20-2. On the battlefield, samples obtained at lower echelons are normally submitted to the local clinical laboratory and proceed through clinical laboratory channels to the 1st or 9th Area Medical Laboratory. Area medical laboratories, descendants of the 520th Theater Army Medical Laboratory, are theater-level tactical laboratories with 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 area medical laboratories have reach-back ability and work closely with national laboratories at USAMRIID and the US Army Medical Research Institute of Chemical Defense.

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.

EXHIBIT 20-2

SAMPLES TO CONSIDER OBTAINING FROM POTENTIAL BIOWARFARE/BIO-TERRORISM 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 in which 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

TABLE 20-2
RECOMMENDED THERAPY FOR (AND PROPHYLAXIS AGAINST) DISEASES CAUSED BY
CATEGORY A BIOTHRREAT 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 Doxycycline 100 mg IV q12h AND one or two additional antimicrobials [§]	Ciprofloxacin [‡] 10–15 mg/kg IV q12h OR Doxycycline 2.2 mg/kg IV q12h AND one or two additional antimicrobials [§]
Anthrax, inhalational, postexposure prophylaxis (60-day course [†])	Ciprofloxacin 500 mg PO q12h OR Doxycycline 100 mg PO q12h	Ciprofloxacin 10–15 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 Doxycycline 100 mg PO q12h	Ciprofloxacin 10–15 mg/kg PO q12h OR Doxycycline 2.2 mg/kg PO q12h
Plague, therapy	Streptomycin 1 g IM twice daily OR Gentamicin 5 mg/kg IV or IM qd OR Doxycycline 100 mg IV or PO q12h OR Ciprofloxacin 400 mg IV or PO q12h	Streptomycin 15 mg/kg IM twice daily OR Gentamicin 2.5 mg/kg IV or IM q8h OR Doxycycline 2.2 mg/kg IV or PO q12h OR Ciprofloxacin 15 mg/kg IV or PO q12h
Plague, 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
Tularemia, therapy, and prophylaxis	Same as for plague	Same as for plague
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.

[†]If the strain is susceptible, children may be switched to oral amoxicillin (80 mg/kg/day q8h) to complete a 60-day course. It is recommended that the first 14 days of therapy or postexposure prophylaxis, however, include ciprofloxacin and/or doxycycline, regardless of age. A three-dose series of Anthrax Vaccine Adsorbed may permit shortening of the antibiotic course to 30 days.

[‡]Levofloxacin or ofloxacin may be acceptable alternatives to ciprofloxacin.

[§]Other antimicrobials with in-vitro activity include rifampin, vancomycin, chloramphenicol, imipenem, clindamycin, or clarithromycin. Doxycycline is not recommended for treating cases with meningoencephalitis due to poor central nervous system penetration.

[¶]Ten 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.

(Table 20-2 continues)

Table 20-2 continued

h: hours; IM: intramuscular; IV: intravenous; q: each, every; qd: every day; PO: by mouth
 Data sources: (1) Centers for Disease Control and Prevention. Update: investigation of bioterrorism-related anthrax and interim guidelines for exposure management and antimicrobial therapy, October 26, 2001. *MMWR Morb Mortal Wkly Rep.* 2001;50:909–919. (2) Centers for Disease Control and Prevention. Update: investigation of anthrax associated with intentional exposure and interim public health guidelines, October 19, 2001. *MMWR Morb Mortal Wkly Rep.* 2001;50:889–893. (3) Centers for Disease Control and Prevention. Notice to readers: additional options for preventive treatment for persons exposed to inhalational anthrax, December 21, 2001. *MMWR Morb Mortal Wkly Rep.* 2001;1142. (4) Inglesby TV, Dennis DT, Henderson DA, et al. Plague as a biological weapon—medical and public health management. *JAMA.* 2000;283:228–2290. (5) Inglesby TV, Dennis DT, Henderson DA, et al. Tularemia as a biological weapon—medical and public health management. *JAMA.* 2001;285:2763–2773. (6) FM 8-284 Working Group. Field Manual 8-284, AFJMAN 44-156, NAVMED P-5042, MCRP 4-11.1C. Treatment of Biological Warfare Agent Casualties. Fort Sam Houston, TX: US Army Medical Department Center and School; 17 July 2000.

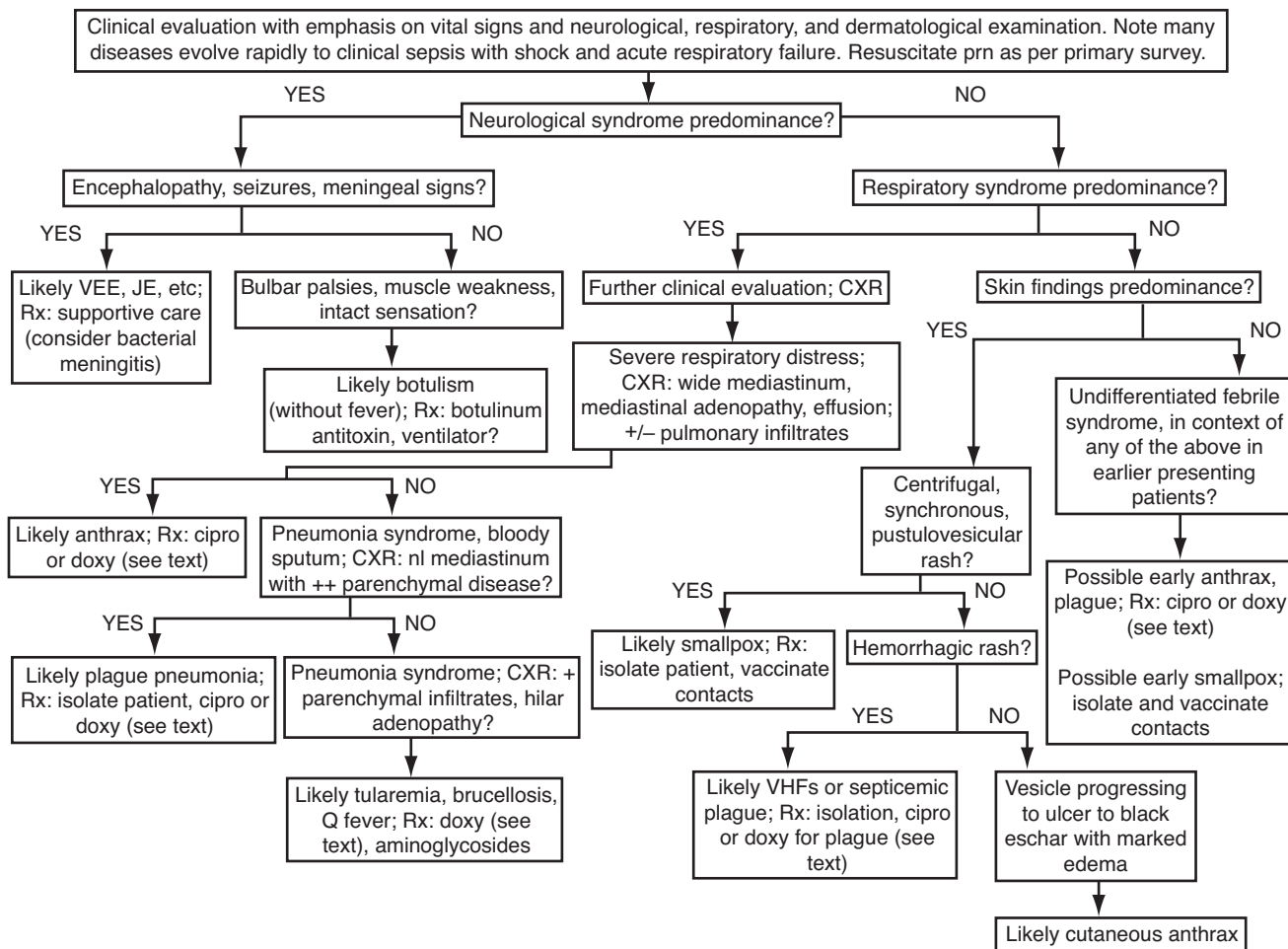


Fig. 20-2. An empiric and algorithmic approach to the diagnosis and management of potential biological casualties. cipro: ciprofloxacin; CXR: chest X-ray; doxy: doxycycline; JE: Japanese encephalitis; nl: normal limits; prn: as needed; Rx: prescription; VEE: Venezuelan equine encephalitis; VHF: viral 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.

Recommendations for this therapy are provided in Table 20-2. When a clinician is faced with multiple patients and the nature of the illness is unknown, empiric therapy must be instituted. Guidelines for providing empiric therapy in these situations have been published,⁷ and an algorithmic approach to syndromic diagnosis and empiric therapy is provided in Figure 20-2. Specifically, doxycycline or ciprofloxacin (Bayer AG, Leverkusen, North Rhine-Westphalia, Germany) should be administered empirically to patients with significant respiratory tract 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, including the following causative agents:

- anthrax,
- tularemia,
- brucellosis,
- Q fever,
- alphaviral equine encephalitides,
- glanders,
- melioidosis, and
- many others (including all of the toxins).

When dealing with these diseases, standard precautions usually suffice.⁴³ More stringent, transmission-based precautions should be applied in certain circumstances. Three subcategories of transmission-based precautions exist:

1. Droplet precautions are required to manage persons with 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.

TABLE 20-3
CONVENTIONAL AND POTENTIAL INFECTIOUS DISEASES: REQUIRED HOSPITAL INFECTION CONTROL PRECAUTIONS*

Standard Precautions (handwashing)	Contact Precautions (gloves and gown [†])	Droplet Precautions (private room [‡] , surgical mask [§])	Airborne Precautions (private room [‡] , negative pressure room, HEPA filter mask)
Anthrax	VRE	Resistant pneumococci	Measles
Botulism	Enteric infections	Pertussis	Varicella
Tularemia	Skin infections	Group A streptococci	Smallpox [¶]
Brucellosis	Lice	Mycoplasma	Certain VHF [¶]
Q fever	Scabies	Adenovirus	– Ebola
Glanders	<i>Clostridium difficile</i> disease	Influenza	– Marburg
Melioidosis	RSV	Pneumonic plague	– Lassa fever
Ricin intoxication	Certain VHF [¶]	Meningococcal disease	Pulmonary TB
SEB intoxication	– Ebola		
T-2 intoxication	– Marburg		
VEE, EEE, WEE	– Lassa fever		
	MRSA		
	Smallpox [¶]		

*Thorough guidelines on hospital infection control can be found in Garner JS. Guidelines for isolation precautions in hospitals. The Hospital Infection Control Practices Advisory Committee. *Infect Control Hosp Epidemiol.* 1996;17:53–80.

[†]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 used as a part of standard and contact precautions (along with eye protection or a face shield) if procedures are likely to generate splashes or sprays of body fluids.

[¶]Indicated for both contact and airborne precautions.

EEE: eastern equine encephalomyelitis; HEPA: high-efficiency particulate air; MRSA: methicillin-resistant *Staphylococcus aureus*; RSV: respiratory syncytial virus; TB: tuberculosis; VEE: Venezuelan equine encephalitis; VHF: viral hemorrhagic fever; VRE: vancomycin-resistant enterococci; WEE: western equine encephalomyelitis

2. Contact precautions should be used to manage certain viral hemorrhagic fever patients.
3. Airborne precautions, ideally including an N-95 HEPA filter mask, should be used to care for persons with smallpox.

A summary of hospital infection control precautions, as they apply to persons affected by biological terrorism, is presented in Table 20-3.

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 can 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, however, to notify preventive medicine officials and laboratory personnel, as well as the Chemical Corps. 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 treatment is most beneficial. Notifying laboratory personnel not only permits them to focus their efforts on diagnosis, but also allows them to take the necessary precautions. Early notification of Chemical Corps personnel allows for battlefield surveillance, detection, and delineation of the limits of contamination. Using M93 "Fox" nuclear, biological, and chemical reconnaissance vehicles (General Dynamics Land Systems [Sterling Heights, Mich]/Thyssen-Henschel [currently integrated into Rheinmetall AG, Dusseldorf, Germany]; Figure 20-3),



Fig. 20-3. The M93 "Fox" nuclear, biological, and chemical reconnaissance vehicle.

personnel can collect soil, water, and vegetation samples; mark areas of contamination; and transmit data to commanders in real time. As the transformation of the US Army progresses, the M93 "Fox" will be replaced by a "Stryker-Platform" NBC Reconnaissance Vehicle, which will also subsume the capabilities and functions of the BIDS system.⁴⁴

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, however, the county represents the lowest governmental entity at which an independent health department exists. In some rural areas lacking county health departments, practitioners would access the state health department directly. Once alerted, local and regional health authorities are knowledgeable about mechanisms for requesting additional support from health officials at higher jurisdictions. Each practitioner should have a point of contact with such agencies and be familiar with mechanisms for contacting them before a crisis arises. A list of useful points of contact is provided in Exhibit 20-3.

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 number of supporting assets and capabilities may be summoned. The National Incident Management System and its component Incident Command System provide a standardized approach to command and control at an incident scene.⁴⁵ Local officials use the Incident Command System when responding to natural and human-caused disasters, and the Incident Command System would be equally applicable in responding to a biological attack. Under the Incident Command System, a designated official, typically the fire chief or the chief of police, serves as local incident commander. The incident commander may have the ability to summon groups of volunteer medical personnel through the Metropolitan Medical Response System, which includes medical strike teams in 125 local jurisdictions. These teams, under contract with mayors of the 125 municipalities, are organized under the Department of Homeland Security's Office of Domestic Preparedness.

In any incident or disaster, whether natural or manmade, 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 officials to make state-level assets (eg, state health departments, state public health laboratories, and state police assets) available. Most state public

health laboratories participate as reference (formerly level B/C) laboratories in the Laboratory Response Network, a collaborative effort of the Association of Public Health Laboratories and CDC. These facilities support hundreds of sentinel (formerly level A) laboratories in local hospitals throughout the nation, and they can provide sophisticated confirmatory diagnosis and typing of biological agents.^{46,47} (An overview of public health laboratory capabilities is provided in Exhibit 20-4. The biosafety-level⁴⁸ precautions used

by these laboratories are outlined in Exhibit 20-5.) State police can provide law enforcement assistance, and state police laboratories can assist with forensic analysis. 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. Virtually every state governor now has one of 55 military Weapons of Mass Destruction-Civil Support teams. 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 can alert the federal coordinating officer, who can, in turn, assist in activating the National Response Plan (see chapter 19 for related information). The National Response Plan 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 National Response Plan, are organized into 15 emergency support functions. Emergency

EXHIBIT 20-3

POINTS OF CONTACT AND TRAINING RESOURCES

Local law enforcement authorities*
 Local or county health department*
 State health department*
 CDC emergency response hotline: 770-488-7100
 CDC Bioterrorism Preparedness and Response Program: 404-639-0385
 CDC emergency preparedness resources:
<http://www.bt.cdc.gov>
 Strategic National Stockpile: Access through state health department
 FBI (general point of contact): 202-324-3000
 FBI (suspicious package information): <http://www.fbi.gov/pressrel/pressrel01/mail3.pdf>
 CBIRF: 301-744-2038
 USAMRIID general information: <http://www.usamriid.army.mil>
 USAMRICD training materials: <http://ccc.apgea.army.mil>
 US Army medical NBC defense information: <http://www.nbc-med.org>
 Johns Hopkins Center for Civilian Biodefense:
<http://www.hopkins-biodefense.org>
 University of Alabama, Birmingham, biodefense education: <http://www.bioterrorism.uab.edu>
 Infectious Diseases Society of America: <http://www.idsociety.org/bt/toc.htm>

*Clinicians and response planners are encouraged to post this list in an accessible location. Specific local and state points of contact should be included.

CDC: Centers for Disease Control and Prevention; CBIRF: Chemical-Biological Incident Response Force; FBI: Federal Bureau of Investigation; NBC: nuclear, biological, and chemical; USAMRICD: US Army Medical Research Institute of Chemical Defense; USAMRIID: US Army Medical Research Institute of Infectious Diseases.

EXHIBIT 20-4

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 to higher-echelon labs within the network.

Reference Laboratories

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

National Laboratories

These laboratories, including those at CDC and USAMRIID, can use BSL-4 practices and serve as the final authority in the workup of bioterrorism specimens. They 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 Diseases

support function 8 provides for health and medical services. Although a specific agency is assigned primary responsibility for each of the 15 emergency support functions, more than two dozen different federal agencies—as well as the American Red Cross—can, under federal law, provide assistance. Federal disaster medical support is primarily the responsibility of the Department of Health and Human Services, although the Office of Emergency Response—a component of the Department of Homeland Security—oversees the National Disaster Medical System.⁴⁹ A principal component of the National Disaster Medical System 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 National Disaster Medical System is its excess hospital bed capacity, held at numerous Department of Veterans Affairs, military, and civilian hospitals throughout the nation.

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 (formerly level

D) laboratories, which support the reference laboratories at the state level and are capable of handling virtually all potential biological threat agents.⁵⁰ Expert consultation and epidemiological investigative assistance are also available through the CDC, and bioweapons threat evaluation and medical consultation are available through USAMRIID. Additionally, the military can provide expert advice and assistance to civilian authorities through the Chemical/Biological Rapid Response Team, which can arrive at a disaster site within a few hours of notification, as well as through the previously described Chemical-Biological Incident Response Force, which is capable of providing reconnaissance, decontamination, and field treatment.⁵¹ Similar to the Chemical/Biological Rapid Response Team, the Chemical-Biological Incident Response Force is trained and equipped to be available within hours of notification. Military support, when provided, is subordinate to civilian authorities. Military support would be provided and tailored by the Joint Task Force for Civil Support (Fort Monroe, Va), a component of US Northern Command (Peterson Air Force Base, Colo), which provides a command-and-control element for all

EXHIBIT 20-5

BIOSAFETY LEVELS

Biosafety Level 1

Involves practices used by a microbiology lab that deals only with well-characterized organisms that do not typically produce disease in humans. Work is conducted on open benchtops using standard microbiological practices. A high school biology lab might use BSL-1 practices.

Biosafety Level 2

Involves practices used by labs that deal with most human pathogens of moderate potential hazard. Lab coats and gloves are typically worn, access to the lab is restricted to trained personnel, and safety cabinets are often used. A clinical hospital laboratory would typically use BSL-2 practices.

Biosafety Level 3

Involves practices used by labs 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 (eg, scrub suits) is exchanged on exiting the lab. Labs are negatively pressurized. A state health department lab would typically use BSL-3 practices.

Biosafety Level 4

Involves practices used by labs 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 and exit the lab through a series of changing and shower rooms. Equipment and supplies enter via a double-door autoclave. Strict and sophisticated engineering controls are used and personnel wear sealed positive pressure space suits with supplied air. Labs are negatively pressurized. Labs at CDC, USAMRIID, the Canadian Science Center for Human and Animal Health, and a few other research facilities are equipped with BSL-4 controls.

BSL: biosafety level

CDC: Centers for Disease Control and Prevention

USAMRIID: US Army Medical Research Institute of Infectious Diseases

military assets involved in disaster response missions and other contingencies within the United States. The CDC has developed the Strategic National Stockpile of critical drugs and vaccines necessary to combat a large disaster or terrorist attack, located at several locations throughout the country and available for rapid deployment to an affected area.⁵² Release of stockpile components is currently controlled by the Department of Homeland Security.

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

The clinician must understand the basic principles of epidemiology and be prepared to assist in the epidemiological investigation after a suspected terrorist attack. Although preventive medicine officers, environmental science officers, veterinarians, epidemiology technicians (91-S in US Army organizations), and field sanitation personnel may be invaluable during an investigation, the clinician should have a working knowledge of the steps involved in an epidemiological investigation. These steps, known as the epidemiological sequence, are published elsewhere⁵³ and summarized in Exhibit 20-6. Although the well-prepared clinician may have a positive impact on 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 initiating an epidemiological investigation and 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.⁵⁴ 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 the consequence of a biological (or chemical or radiological) attack. Symptoms from anxiety and autonomic arousal, as well as side effects from postexposure to prophylactic drugs, may mimic prodromal disease from biological agent exposure and pose dilemmas in differential diagnosis. Persons with symptoms arising from naturally occurring infectious diseases may 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. Communication should include an assessment of the risk of exposure, information on 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.⁵⁵ Readily accessible (biological, chemical, and radiological), agent-specific information packages for local public health authorities and the general public are available through the CDC, and they can be of valuable assistance in risk communication.⁵⁶

Effective risk communication is possible only in the presence of well-conceived risk communication plans and tactics that are worked out well in advance of an actual event. Similar advanced planning must consider the need to rapidly establish local centers for the initial evaluation and administration of post-exposure prophylaxis. 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 Plan³³ provides a useful template for a coordinated, multifaceted approach. The wisdom of farsighted planning and coordination was amply demonstrated by the efficient mass prophylaxis of more than 10,000 individuals in New York City during the events surrounding the discovery of anthrax-contaminated mail in 2001.⁵⁷

EXHIBIT 20-6

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 3030-G*. 2nd ed. Atlanta, Ga: CDC; 1998: 347-424.

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 the planning and execution of conventional field training and command post exercises. In the future, however, these 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 a necessary component of disaster preparation. These exercises should be designed 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 evalu-

ation in a disaster drill or field-training exercise. The Joint Commission on the Accreditation of Healthcare Organizations requires hospitals to conduct a hazard vulnerability analysis, develop an emergency management plan, and evaluate this plan twice yearly; one of these evaluations must include a communitywide drill.⁵⁸ Moreover, the Joint Commission on the Accreditation of Healthcare Organizations specifically mandates that hospitals provide facilities (and training in the use of such facilities) for radioactive, biological, and chemical isolation and decontamination.

Many resources, including this textbook, 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. Moreover, electronic resources of a similar nature have been developed^{59,60} and multiple Web sites provide a wealth of training materials and information on-line⁶¹ (see Exhibit 20-3) to assist military and civilian clinicians and public health professionals.

SUMMARY

To help manage the casualties that may result from biological warfare or terrorism, USAMRIID has developed a 10-step approach that specifies a tactical response as well as operational and strategic response. Military and civilian clinicians and public health professionals must be proficient in and plan for real or threatened terror attacks. Numerous governmental, military, and ci-

vilian organizations have now been organized, trained, and equipped to provide assistance and consultation to the clinician, first responder, and public health official 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 21

MEDICAL COUNTERMEASURES

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INTRODUCTION

BACTERIAL AND RICKETTSIAL DISEASES

- Anthrax
- Tularemia
- Plague
- Glanders and Melioidosis
- Brucellosis
- Q Fever

VIROLOGY

- Alphaviruses
- Smallpox
- Viral Hemorrhagic Fevers

TOXINS

- Botulinum Toxin
- Staphylococcal Enterotoxin B
- Ricin

SUMMARY

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INTRODUCTION

Countermeasures against bioterrorism 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 initial three countermeasures are non-medical and discussed in other chapters. This chapter will be restricted to medical countermeasures, which

include interventions such as active immunoprophylaxis (ie, vaccines), passive immunoprophylaxis (ie, immunoglobulins and antitoxins), and chemoprophylaxis (ie, postexposure antibiotic prophylaxis) (Tables 21-1 and 21-2). Medical countermeasures may be initiated either before an exposure (if individuals are identified as being at high risk for exposure) or after a confirmed exposure event. Because medical countermeasures

TABLE 21-1
VACCINES, VACCINE DOSAGE SCHEDULES, AND POSTVACCINATION PROTECTION

Vaccine	Primary Series	Protection	Booster Doses
Anthrax (0.5 mL SQ)	Days 1, 14, 28 Months 6, 12, 18	3 weeks after 3rd vaccine dose	Annual boosters after dose 6 of vaccine
Tularemia* [†] (15 punctures PC)	Day 0	“Take” after vaccination	Every 10 years [†]
Q fever [‡] (0.5 mL SQ)	Day 0	3 weeks after vaccination	None
VEE C-83* [§] (0.5 mL SQ)	Day 0	Titer ≥ 1:20	None (boost with TC-84) [¶]
VEE TC-84 [§] (0.5 mL SQ)	Day 0	Titer ≥ 1:20	As needed per titer [¶]
EEE [¶] (0.5 mL SQ)	Days 0, 7, 28	Titer ≥ 1:40	As needed per titer [¶]
WEE [¶]	Days 0, 7, 28	Titer ≥ 1:40	As needed per titer [¶]
Yellow fever* (0.5 mL SQ)	Day 0	4 weeks after vaccination	Every 10 years
Smallpox*** (3 punctures PC for primary vaccination)	Day 0	Evidence of a “take” (vesiculo-papular response); Scab resolved (day 21-28 after vaccination)	1, 3, or 10 years**
RVF (1 mL SQ)	Days 0, 7, 28, 180	Titer ≥ 1:40 after dose 3	As needed per titer [¶]
Junin* ^{††} (0.5 mL IM)	Day 0	4 weeks after vaccination	None
TBE ^{§§} (0.5 mL SQ)	Days 0, 30	2 weeks after 2nd vaccine dose	Every 3 years
PBT ^{¶¶} (0.5 mL SQ)	Days 0, 14, 84, and month 6	Potential protection within 4 weeks of 3rd vaccine dose (antitoxin titers no longer obtained)	Booster dose at 12 months and then yearly

* Live vaccine.

[†] Investigational live attenuated tularemia NDBR 101 vaccine. Booster doses currently recommended every 10 years, although immunity may persist longer.

[‡] Investigational inactivated freeze-dried Q Fever NDBR 105 vaccine.

[§] Investigational live attenuated TC-83 NDBR 102 VEE vaccine is given as a one-time injection. PRNT₈₀ titers were obtained after vaccination and yearly to assess for adequate titers. If PRNT₈₀ titers fell below a predetermined level, another investigational vaccine, the inactivated C-84 TSI-GSD-205 VEE vaccine, was given to boost titers.

[¶] PRNT₈₀ titers. Titers are obtained within 28 days of the primary series and yearly afterward to assess immune response. Booster doses for EEE were administered as 0.1 mL intradermally.

^{¶¶} Investigational inactivated TSI-GSD-104 EEE and TSI-GSD-210 WEE vaccines.

**Booster doses are administered as 15 punctures PC, given every 10 years, but may be recommended more frequently if high risk of exposure (ie, smallpox outbreak, laboratory workers). Laboratory workers are given booster doses every 3 years if working with monkeypox and yearly if working with variola (variola research only at CDC).

^{††} Investigational live attenuated AHF virus vaccine (Candid 1).

^{§§} Investigational FSME-IMMUN inject vaccine.

^{¶¶} Investigational botulinum pentavalent (ABCDE) botulinum toxoid.

CDC: Centers for Disease Control and Prevention; EEE: eastern equine encephalitis; IM: intramuscular; MA: microagglutination titer; PBT: pentavalent botulinum toxoid; PC: percutaneous; PRNT₈₀: 80% plaque reduction neutralization titer; RVF: Rift Valley fever; SQ: subcutaneous; TBE: tick-borne encephalitis; VEE: Venezuelan equine encephalitis; WEE: western equine encephalitis

TABLE 21-2
POSTEXPOSURE ANTIBIOTIC PROPHYLAXIS REGIMENS

Agent	Antibiotic	Duration of Treatment
<i>Bacillus anthracis</i> *	Ciprofloxacin, doxycycline, or penicillin (if sensitive)	Vaccinated: 30 days (aerosol) Unvaccinated: 60 days (aerosol)
<i>Yersinia pestis</i>	Doxycycline or ciprofloxacin	7 days
<i>Francisella tularensis</i>	Doxycycline or ciprofloxacin	14 days
<i>Burkholderia mallei</i>	Doxycycline, trimethoprim-sulfamethoxazole, augmentin, or ciprofloxacin	14 days (consider 21 days) [†]
<i>B pseudomallei</i>	Doxycycline, trimethoprim-sulfamethoxazole (possibly ciprofloxacin)	14 days (consider 21 days) [†]
<i>Brucella</i>	Doxycycline plus rifampin	21 days
<i>Coxiella burnetii</i>	Doxycycline	7 days (not to be given before day 8 after exposure because it may only prolong the incubation period)

* Advisory Committee on Immunization Practices membership notes no data on postexposure prophylaxis for preventing cutaneous anthrax but suggests 7- to 14-day course of antibiotics may be considered.

[†]No clinical data to support

may be associated with adverse events, the recommendation for their use must be weighed against the risk of exposure and disease. Vaccines, both investigational and approved by the Food and Drug Administration (FDA), are available for some bioterrorism agents. In the event of a bioterrorist 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 bioterrorist agent, particularly with vaccinations that require booster doses to maintain immunity. In these cases, chemoprophylaxis after identifying an exposure may be effective in preventing disease. Any effective bioterrorism plan should address the logistics of maintaining adequate supplies of drugs and vaccines, as well as personnel to coordinate and dispense needed supplies to the affected site.

Although the anthrax and smallpox vaccines are both FDA approved, potential bioterrorism agents have only investigational vaccines that were developed and manufactured over 30 years ago. 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 these vaccines can be obtained under investigational new drug (IND) protocols at limited sites in the United States, the vaccines are in extremely limited supply and are declining in immunogenicity with age.

Under the FDA animal rule instituted in 2002, approval of vaccines can now be based on demonstration of efficacy in animal models alone, if efficacy studies in humans would be unsafe or unethical. This rule has opened the opportunity to develop many new and improved vaccines, with the ultimate goal of FDA licensure. Vaccine development generally is a long process, requiring 3 to 5 years to identify a potential vaccine candidate and conduct animal studies to test for vaccine immunogenicity and efficacy, with an additional 5 years of clinical trials for FDA approval and licensure. FDA vaccine approval then takes from 7 to 10 years, so 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. Associated disease may occur in wildlife such as deer and bison in the United States but occurs most frequently in domestic animals such as sheep, goats, and cattle, which acquire

spores by ingesting contaminated soil. Humans can become infected through skin contact, ingestion, or inhalation of *B anthracis* spores from infected animals or animal products. Anthrax is not transmissible from person to person. The infective dose for inhalational anthrax based on nonhuman primate studies is estimated to be 8,000 to 50,000 spores.^{1,2} The 2001 anthrax

incident suggests that inhalational anthrax may result from inhalation of relatively few spores with exposure to small particles of aerosolized anthrax.³ The stability and prolonged survival of the spore stage makes *B anthracis* an ideal agent for bioterrorism.

Vaccination

History of the anthrax vaccine. In 1947 a factor isolated from the edema fluid of cutaneous *B anthracis* lesions was noted to successfully vaccinate animals.⁴ This factor, identified as the protective antigen (PA), was subsequently recovered from incubating *B anthracis* in special culture medium.^{5,6} This 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*.⁷

This early version of the anthrax vaccine was demonstrated to protect small laboratory animals⁸ and nonhuman primates from inhalational anthrax.⁷ The vaccine also demonstrated protection against cutaneous anthrax infections in employees working in textile mills processing raw imported goat hair.⁸ During this study, only 3 cases of cutaneous anthrax occurred in 379 vaccinated employees, versus 18 cases of cutaneous anthrax and all 5 cases of inhalational anthrax that occurred in the 754 nonvaccinated employees. Based on these results, the vaccine efficacy for anthrax was determined to be 92.5%. The vaccine failures were noted in a 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 (infection at 13 months), and a third person who was within a week of the fourth vaccine dose (the 6-month dose), a period when titers are known to be lower. Local reactions were noted in 35% of vaccinees, but most reactions were short-lived (generally resolving within 24 to 48 hours), with severe reactions occurring in only 2.8% in the vaccinated population.

Anthrax vaccine adsorbed. The current FDA-approved anthrax vaccine adsorbed (AVA) was derived through improvements of the early alum-precipitated anthrax vaccine and involved (a) using a *B anthracis* strain that produced a higher fraction of PA, (b) growing the culture under microaerophilic instead of aerobic conditions, and (c) substituting an aluminum hydroxide adjuvant in place of the aluminum potassium salt adjuvant.^{9,10} Originally produced by the Michigan Department of Public Health, AVA is now manufactured by BioPort Corporation in Lansing, Michigan. 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 relative absence of other toxin components such as lethal factor or edema factor.^{9,11} 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.¹¹

AVA is given as subcutaneous injections (in the upper deltoid muscle) of 0.5 mL at 0, 2, and 4 weeks, followed by injections at 6, 12, and 18 months, and then yearly boosters. Vaccine breakthroughs have been reported in persons who received only two doses of vaccine, but infections in those who received all three initial doses (and are current on subsequent primary and booster doses) are uncommon. The few published reports of breakthroughs occurred with use of the earlier, alum-precipitated anthrax vaccine and within days before the scheduled 6-month vaccine dose (dose 4), when antibody titers have been demonstrated to be low.^{8,12}

Evidence suggests that both humoral and cellular immune responses against PA are critical to protection against disease after exposure.^{9,13,14} 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.¹⁵ Approximately 95% of vaccinees seroconvert with a 4-fold rise in anti-PA IgG titer after three doses of vaccine.^{13,16} Although animal studies have demonstrated transfer of passive immunity from polyclonal antibodies,¹⁷ the correlation of protection against anthrax infection with a specific antibody titer has not yet been defined.¹³

Both the alum-precipitated vaccine and AVA demonstrated efficacy in animal models against aerosol challenge.^{6,7,10,13-15,18-20} A total of 52 of 55 monkeys (95%) given two doses of anthrax vaccine survived lethal aerosol challenge without antibiotics.²¹ Because spore forms of *B anthracis* may persist for over 75 days after an inhalational exposure, vaccination against anthrax may provide more prolonged protection than post-exposure antibiotic prophylaxis alone.^{22,23} However, vaccination after exposure alone was not effective in preventing disease from inhalational anthrax. Vaccination of rhesus monkeys at days 1 and 15 after aerosol exposure did not protect against inhalational anthrax (4×10^5 spores, which is 8 median lethal doses) resulting in death in 8 of the 10 monkeys. However, all rhesus monkeys given 30 days of doxycycline in addition to postexposure vaccination survived.²⁴ Recent studies indicate that a short course of postexposure antibiotics (14 days) in conjunction with vaccination provides

significant protection against high dose aerosol challenge in nonhuman primates.²⁵

Vaccine adverse events. Adverse reactions in 6,985 persons who received a total of 16,435 doses of AVA (9,893 initial series doses and 6,542 annual boosters) were primarily local reactions.²⁶ Local reactions (edema or induration) were severe (> 12 cm) in less than 1% vaccinations, moderate (3–12 cm) in 3% vaccinations, and mild (< 3 cm) in 20% vaccinations. Systemic reactions were uncommon, occurring in less than 0.06% of vaccines, and included fever, chills, body aches, or nausea.

Data from the Vaccine Adverse Event Reporting System from 1990 to 2000, after nearly 2 million doses of vaccine were distributed, showed approximately 1,500 adverse events reported from the vaccine. The most frequently reported events were injection site hypersensitivity (334), edema at the injection site (283), pain at the injection site (247), headache (239), arthralgia (232), asthenia (215), and pruritus (212). Only 76 events (5%) were serious, including the reporting of anaphylaxis in two cases.²⁷

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, females were found to be more likely than males to have injection site reactions, edema, and lymphadenopathy.²⁸ Initial data also showed a decrease in the rate of local reactions if the time interval between the first and second dose was extended or if the vaccine was administered intramuscularly. No decrease in seroconversion rates or anti-PA IgG geometric mean titers was noted with either of these modifications of administration. Delay of the second vaccine dose to 4 weeks (instead of 2 weeks) was associated with induration in only 1 of 10 females (10%) and subcutaneous nodules in only 4 of 10 females (40%), versus 10 of 18 (56%) and 15 of 43 (83%), respectively, when the second vaccine dose was given at 2 weeks.²⁹ When AVA was administered intramuscularly at 0 and 4 weeks, none of the 10 persons exhibited induration or subcutaneous nodules, and only one person developed erythema. The Centers for Disease Control and Prevention (CDC) is conducting a large study to confirm these results.

Protocols for managing vaccine adverse events 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. In special cases, to alleviate future discomfort for patients with large or persistent injection-site reactions after subcutaneous

injection, the US Army Medical Command policy for troops allows intramuscular injection to be considered if the provider (a) believes intramuscular injection will provide appropriate protection and reduce side effects, and (b) informs the patient that intramuscular injection is not FDA approved.³⁰

Additional anthrax vaccination is contraindicated in persons who have experienced an anaphylactic reaction to the vaccine or any of the vaccine components.²² It is also contraindicated in persons with a history of anthrax infection because of previous observations of an increase in severe adverse events.²² The vaccine may be given in pregnancy only if the benefit outweighs the risk.

Other anthrax vaccines. An attenuated live anthrax vaccine given by scarification or subcutaneous injection is used in the former Soviet Union. The vaccine 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.^{31,32} A PA-based anthrax vaccine, made by alum precipitation of a cell-free culture filtrate of a derivative of the attenuated *B anthracis* Sterne strain, is currently licensed in the United Kingdom.^{19,33}

New vaccine research. The ability to prepare purified components of anthrax toxin by recombinant technology has presented the possibility of new anthrax vaccines. New vaccine candidates may be PA toxoid vaccines or PA-producing live vaccines that elicit partial or complete protection against anthrax infection.¹⁹ A recombinant PA vaccine candidate given intradermally or intranasally was demonstrated to provide complete protection in rabbits and nonhuman primates against aerosol challenge with anthrax spores.³⁴

Recent research has shown toxin neutralization approaches to be protective in animal models. Inter-alpha inhibitor protein (I α Ip), an endogenous serine protease inhibitor in human plasma, given to BALB/c mice 1 hour before intravenous challenge to a lethal dose of *B anthracis*, was associated with a 71% survival rate at 7 days compared to no survivors in the control groups.³⁵ One potential mechanism of action for I α Ip is through the inhibition of furin, an enzyme required for assembling lethal toxin in anthrax pathogenesis.

Chemoprophylaxis

Antibiotics. Antibiotics are effective only against the vegetative form of *B anthracis* (not effective against the spore form). However, in the nonhuman primate model of inhalational anthrax, spores have been shown to survive for months (< 1% at 75 days and trace spores present at 100 days) without germination.²²⁻²⁴

Prolonged spore survival has not been observed for other routes of exposure.

Ciprofloxacin, doxycycline, and penicillin G procaine have been FDA approved for prophylaxis of inhalational anthrax.^{2,11,22,24,36} Ciprofloxacin, doxycycline, and penicillin have been demonstrated in nonhuman primates to reduce the incidence or progression of disease after aerosol exposure to *B anthracis*.^{22,24,36} Macaques exposed to 240,000 to 560,000 anthrax spores (8 median lethal doses) and given postexposure antibiotic prophylaxis with 30 days of penicillin, doxycycline, or ciprofloxacin resulted in survival of 7 of 10, 9 of 10, and 8 of 9 monkeys, respectively.²⁴ 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 day 58 postexposure, and one monkey treated with ciprofloxacin died day 36 postexposure. This phenomenon is attributed to delayed vegetation of spores that may persist in lung tissue after inhalational exposure.

To avoid toxicity in children and pregnant or lactating women exposed to penicillin-susceptible strains, amoxicillin given three times daily is an option. However, it is not recommended as a first-line treatment because it lacks FDA approval and its efficacy and ability to achieve adequate therapeutic levels at standard doses are uncertain. Because strains may be resistant to penicillin, amoxicillin should not be used until sensitivity testing has been performed.²²

Duration of antibiotic prophylaxis. The optimal duration of postexposure antibiotic prophylaxis after aerosol exposure to *B anthracis* in unvaccinated individuals is 60 days, which is based on the results of the animal studies described above.^{22,24,37} Spore survival in the lung tissue of Macaques exposed to 4 median lethal doses was estimated to be 15% to 20% at 42 days, 2% at 50 days, and less than 1% at 75 days.²²⁻²⁴ The 1979 outbreak of inhalational anthrax after an accidental release of spores from a Soviet biological weapons production facility (the Sverdlovsk outbreak) suggests that lethal spores persisted after the initial exposure because cases of human anthrax developed as late as 43 days after the release.³⁸ Current recommendations for treating unvaccinated persons after aerosol exposure to *B anthracis* from the CDC, Advisory Committee for Immunization Practices (ACIP), and Occupational Safety and Health Administration, are for 60 days of either ciprofloxacin (500 mg twice daily) or doxycycline (100 mg twice daily).^{22,37} Tetracycline may be a possible alternative for doxycycline, but it has not been well studied.

Adverse events of chemoprophylaxis. Adverse events associated with the prolonged, 60-day, antibiotic prophylaxis regimen have had a significant impact on

compliance. Compliance was reported to be as low as 42% among the 10,000 persons in the 2001 incident at the Brentwood Post Office and Senate office building who were recommended to receive the regimen.³⁹ Adverse events reported by the 3,428 postal workers receiving postexposure prophylaxis with ciprofloxacin were primarily gastrointestinal symptoms of nausea, vomiting, or abdominal pain (19%); fainting, dizziness, or light-headedness (14%); heartburn or acid reflux (8%); and rash, hives, or itchy skin (7%).⁴⁰ Reasons for early discontinuation of ciprofloxacin included adverse events (3%), fear of possible adverse events (1%), and belief that the drug was unnecessary (1%). Other adverse events that can occur with quinolones but not reported in this survey include headache, tremors, restlessness, confusion, and Achilles tendon rupture.⁴⁰ Adverse events associated with tetracycline and amoxicillin were predominantly gastrointestinal symptoms.

Postexposure Vaccination With Chemoprophylaxis

Vaccination alone after exposure to *B anthracis* was not protective in preventing inhalational anthrax in nonhuman primates; therefore, AVA is not currently licensed for postexposure prophylaxis. Both the ACIP and CDC endorse making anthrax vaccine available for unvaccinated persons identified as at risk for inhalational exposure in a three-dose regimen (0, 2, and 4 weeks) in combination with antimicrobial postexposure prophylaxis under an IND application.⁴¹ However, there is insufficient data to determine the duration of antibiotic prophylaxis when initiated with vaccination. Based on antibody titers peaking at 14 days after the third dose of AVA,⁴² a recommendation of 30 days was suggested in persons already fully or partially immune, and perhaps 7 to 14 days after the third vaccine dose when the vaccine was initiated in conjunction with postexposure prophylaxis. Doxycycline given for 30 days after aerosol exposure resulted in survival of 9 of 10 monkeys, and doxycycline given for 30 days after aerosol exposure in conjunction with two doses of anthrax vaccine was protective in 9 of 9 monkeys challenged with *B anthracis*.²⁴ The addition of the vaccine may suggest a possible benefit, but the difference was not statistically different ($P = 0.4$) for this study.²⁴ However, recent nonhuman primate studies indicated that a 14-day course of oral ciprofloxacin in combination with AVA vaccination may significantly reduce the duration of postexposure prophylaxis, from 30 days to 14 days with a statistical significance of $P = 0.011$.²⁵ In this study, vaccine was provided on days 0, 14, and 30, with 100% protection (10/10) of nonhuman primates receiving a 14-day course of oral

ciprofloxacin and three doses of AVA vaccine. Because there are no prolonged spore stages with percutaneous and gastrointestinal exposures, the CDC does not recommend postexposure prophylaxis in these instances. However, the ACIP noted that there are no controlled studies of this issue and suggested a course of 7 to 14 days as prophylaxis for both cutaneous and gastrointestinal anthrax provided no inhalational exposure is suspected.^{41,43}

Clinical Indications for Vaccine or Postexposure Antibiotic Prophylaxis

Evaluation for inhalational exposure to *B anthracis* includes a physical examination, laboratory tests, and chest radiograph, as indicated, to exclude active infection. Nasal swabs may be used for epidemiological purposes, but should not be used as a primary determinate for the initiation or cessation of postexposure antibiotic prophylaxis^{44,45}; a negative nares culture does not exclude inhalational exposure to the organism. However, if an individual has a positive nares culture, postexposure antibiotic prophylaxis should be initiated.

Antibiotic prophylaxis should be initiated upon possible aerosol exposure to *B anthracis* and should be continued until *B anthracis* exposure has been excluded. If exposure is confirmed or cannot be excluded, prophylaxis should continue for 60 days duration in unvaccinated persons. In unvaccinated individuals who subsequently undergo vaccination, antibiotic prophylaxis should be continued for 7 days after the third dose of vaccine is administered. For persons with a history of anthrax vaccination who are within 1 year of their annual booster, a 30-day course of antibiotics should be sufficient. Individuals should be monitored for symptoms throughout the incubation period, lasting 1 to 7 days after percutaneous exposure or ingestion, and potentially up to 90 days following aerosol exposures.

Tularemia

Francisella tularensis, a highly infectious bacterial pathogen responsible for serious illness, and occasionally death, has long been recognized as a potential biological weapon.⁴⁶ 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 (deerflies, mosquitoes, or ticks); (c) ingestion of contaminated food or water (less commonly); or (d) inhalation of aerosolized agent from infected animal secretions. Tularemia is not transmissible person to person. Because of the low infective dose (10–50 organisms) of *F tularensis*, disease may

readily develop when exposure is by the pulmonary route. This disease was the most common laboratory-acquired infection (153 cases) during the 25 years of the US Biological Warfare Program. These tularemia infections were acquired mainly from aerosol exposures.¹² Outbreaks of tularemia in nonendemic areas should alert officials to the possibility of a bioterrorism event.

Vaccination

Investigational live tularemia vaccine. No FDA-licensed vaccine protecting against tularemia is currently available. However, an investigational live attenuated vaccine given to at-risk researchers at Fort Detrick, Maryland, has been available since 1959. This vaccine is only available at USAMRIID under an IND protocol.

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.^{47–49} A sample of the Soviet live tularemia vaccine (known as strain 15), which was used in millions of persons during epidemics of type B tularemia beginning in the 1930s, was made available to Fort Detrick in 1956.⁴⁸ Both a gray-variant and blue-variant colony were cultivated from this vaccine (colonies were blue when illuminated with oblique light under a dissecting microscope). The blue-variant colony was proven to be both more virulent and more immunogenic than the gray-variant colony. To improve protection against the virulent *F tularensis* SCHU S4 strain, the blue-variant colony was passaged through white mice to potentiate its virulence and immunogenicity. These passages subsequently 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.

Beginning in 1959, the live attenuated tularemia vaccine, LVS, was administered to at-risk laboratory personnel in the offensive biological warfare program at Fort Detrick until closure of the program in 1969 (Figure 21-1).⁴⁷ Before vaccination, tularemia was the most frequently diagnosed laboratory-acquired infection, with mainly typhoidal/pneumonic and ulceroglandular disease manifestations. After vaccination, the incidence of typhoidal/pneumonic tularemia decreased from 5.7 to 0.27 cases per 1,000 at-risk employee-years. Although no decrease in ulceroglandular tularemia was noted during this time, the vaccine did ameliorate symptoms from ulceroglandular tularemia,

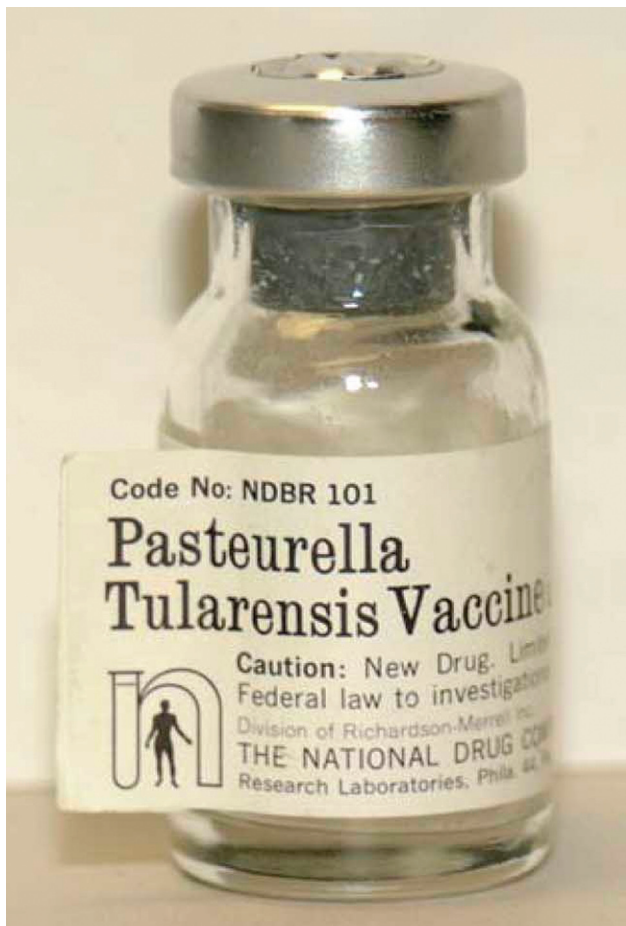


Fig. 21-1. Live attenuated NDBR 101 tularemia vaccine. Vaccination of at-risk laboratory workers, beginning in 1959, resulted in a decreased incidence of typhoidal tularemia from 5.7 to 0.27 cases per 100 at-risk employee-years, and ameliorated symptoms from ulceroglandular tularemia. The vaccine is administered by scarification with 15 to 30 pricks on the forearm, using a bifurcated needle.

and vaccinated persons no longer required hospitalization. The occurrence of ulceroglandular tularemia in vaccinated persons was consistent with the observation that natural disease also failed to confer immunity to subsequent infections of ulceroglandular tularemia. In 1961 commercial production of LVS was initiated by the National Drug Company, Swiftwater, Pennsylvania, under contract to the US Army Medical Research and Materiel Command; this vaccine was designated NDBR 101. The vaccine continues to be given as an investigational drug to at-risk laboratory workers in the US Biodefense Program.

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,” 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 generally seen in vaccinia vaccinations. Although a take is related to immunity, its exact correlation has not yet been determined (Figure 21-2). Studies measuring cell-mediated immunity to tularemia in vaccinees are being undertaken to determine the duration of immunity from the vaccine.

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.^{50,51} Cell-mediated immunity responses occur within 1 to 4 weeks after naturally occurring infection or after LVS vaccination and reportedly last a long time (10 years or longer).^{50,52-59} 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 the unvaccinated control group.⁶⁰ A similar experience was observed in



Fig. 21-2. “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.

studies of the inactivated Foshay tularemia vaccine, in which antibodies were induced by the vaccine but were not protective against tularemia.^{47,49} Although nearly all vaccinees develop a humoral response, with microagglutination titers appearing between 2 and 4 weeks postvaccination,^{50,57,61} a correlation could not be demonstrated between antibody titers and the magnitude of lymphocyte proliferative responses.^{51,59,62,63} 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.⁶²

Vaccine adverse events. The local skin lesion after vaccination (known as a 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 form a scab lasting approximately 2 to 3 weeks. Local axillary lymphadenopathy is not uncommon, reported in 20% to 36% of persons. 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 not determined to be vaccine related. The main contraindications of the vaccine are prior tularemia infection, immunodeficiency, liver disease, and pregnancy.

Other vaccines. The current US IND tularemia vaccine was derived from the Soviet live attenuated vaccine dating from the 1930s. Research is ongoing to develop a new LVS tularemia vaccine (using the National Drug Company's LVS as a starting material) as well as subunit vaccines against tularemia.⁶⁴

Chemoprophylaxis

Prophylaxis with tetracycline given as a 1-g dose twice daily within 24 hours of exposure for 14 days was demonstrated to be highly effective for preventing tularemia in humans exposed to aerosols of 25,000 *F tularensis* SCHU-S4 spores, with none of the eight exposed persons becoming ill.⁶⁵ 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 due to considerable fluctuations in tissue levels, as demonstrated in monkeys given once daily tetracycline, which ameliorated symptoms but did not prevent tularemia.⁶⁵

Whereas streptomycin for 5 days successfully prevented tularemia in humans after intradermal challenge with an inoculation of *F tularensis*, neither chlor-

amphenicol nor tetracycline given in a 5-day course was effective as postexposure prophylaxis.⁶⁶ *F tularensis* is an intracellular pathogen that is cleared slowly from the cells, even in the presence of bacterostatic antibiotics. Tetracyclines, even in high concentrations, merely suppress multiplication of the organisms,⁶⁴ which may explain the requirement for a prolonged 14-day course of bacterostatic antibiotics.

Based on the above studies, 100 mg of doxycycline orally twice a day or 500 mg of tetracycline orally four times a day for 14 days is recommended for postexposure prophylaxis to *F tularensis*. A 500-mg dose of ciprofloxacin orally twice a day may be considered as an alternative regimen.

Plague

Plague is an acute bacterial disease caused by a non-motile, gram-negative bacillus known as *Yersinia pestis*.⁶⁷ Naturally occurring disease is generally acquired from bites of infected fleas, resulting in lymphatic and blood infections (bubonic and septicemia plague). Less commonly, plague may occur from direct handling of skins of dead animals, by inhalation of aerosols from infected animal tissues, or by ingestion of infected animal tissues. Pneumonic plague may be acquired by inhaling droplets emitted from an infected person or by inhaling *Y pestis* as an aerosolized weapon, or it may occur as a result of secondary hematogenous seeding from plague septicemia. As the causative agent of pneumonic plague, *Y pestis* is a candidate for use as biological warfare or terrorism agent, with symptoms occurring within 1 to 4 days after aerosol exposure.

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 demonstrated efficacy in the prevention or amelioration of bubonic plague based on retrospective indirect evidence in vaccinated military troops, it had not been proven effective for pneumonic plague.⁶⁸⁻⁷⁵ Vaccine efficacy against aerosolized plague was demonstrated to be poor in animal models, with at least two persons developing pneumonic plague despite vaccination.⁶⁹⁻⁷⁵

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.⁷⁰ For safety reasons, this vaccine is not used for humans in most countries.

New vaccine research. Because of safety issues with live vaccine, recent efforts have focused on the development of a subunit vaccine using virulence factors from the surface of the plague bacteria to induce immunity.^{69,76} Two virulence factors were found to induce immunity and provide protection against plague in animal models, identified as the fraction 1 (F1) capsular antigen and the virulence (V) antigen. At USAMRIID the first new plague vaccine was developed by fusing the F1 capsular antigen with the V antigen to make the recombinant F1-V vaccine. The F1-V vaccine candidate has been shown to be protective in mice and rabbits against both pneumonic and bubonic plague. In nonhuman primates during aerosol challenge experiments, it provided better protection than either the F1 or V antigen alone.^{77,78}

Chemoprophylaxis

Postexposure prophylaxis with ciprofloxacin for 5 days was highly effective as prophylaxis in mice, when administered within 24 hours after aerosol exposure.^{79,80} However, if ciprofloxacin was administered after the onset of disease, approximately 48 hours postexposure, most studies resulted in high rates of treatment failure.^{79,80} Doxycycline was relatively ineffective as prophylaxis in one mouse model study, even if given within 24 hours after aerosol exposure with mean inhibitory concentrations (MICs) ranging from 1 to 4 mg/L.^{79,80} The effectiveness of doxycycline, a bacterostatic drug, generally requires antibiotic levels to be 4 times the MIC. The treatment failure may be related in part to increased metabolism of doxycycline in mice, because tetracycline has been used successfully in humans to treat or prevent pneumonic plague and because doxycycline was able to stabilize the bacterial loads in spleens of mice infected with *Y pestis* strains with lower MICs (≤ 1 mg/L).⁸¹

Recommendations for postexposure prophylaxis after a known or suspected *Y pestis* exposure are doxycycline (100 mg twice daily), tetracycline (500 mg four times daily), or ciprofloxacin (500 mg twice daily) for 7 days or until exposure has been excluded.^{67,79,80,82,83} Postexposure prophylaxis should be given to persons exposed to aerosols of *Y pestis* and to close contacts of persons with pneumonic plague (within 6.5 feet). It should be administered as soon as possible because of the short incubation of plague (1 to 4 days). Sulfonamides have been used in the past to successfully treat plague, but they are less effective than tetracycline and are not effective against pneumonic plague. Therefore, use of trimethoprim-sulfamethoxazole (TMP-SMZ) (1.6–3.2 g of the trimethoprim component per day given twice daily) has been suggested for prophylaxis only in persons with contraindications to tetracyclines

or ciprofloxacin.⁸⁴ Chloramphenicol (25 mg/kg orally four times a day) is an alternative in individuals who cannot take tetracyclines or quinolones, but has the risk of aplastic anemia.⁶⁷ Antibiotic sensitivity testing should be performed to assess for resistant strains.

Glanders and Melioidosis

Glanders and melioidosis are zoonotic diseases caused by gram-negative bacteria, *Burkholderia mallei* and *B pseudomallei*, respectively.^{85–87} The natural reservoirs for *B mallei* are equines. Infection with *B mallei* in horses may be systemic with prominent pulmonary involvement (known as glanders), or may be characterized by subcutaneous ulcerative lesions and lymphatic thickening with nodules (known as farcy). Glanders in humans is not common and has generally been associated with contact with equines. 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, with onset of symptoms 10 to 14 days after aerosol exposure.

B pseudomallei is a natural saprophyte that can be isolated from soil, stagnant waters, rice paddies, and market produce in endemic areas such as Thailand. Infection in humans is generally acquired through soil contamination of skin abrasions, but may also be acquired from ingesting or inhaling the organism. Although symptoms of *B pseudomallei* infection are variable, the pulmonary form of the disease is the most common and may occur as a primary pneumonia or from secondary hematogenous seeding. The incubation period may be as short as 2 days, but the organism may remain latent for a number of years before symptoms occur. Both *B mallei* and *B pseudomallei* have been studied in the past as potential biowarfare agents, and the recent increase of biodefense concerns has renewed research interest in these organisms.

Vaccination

No vaccines are currently available for preventing glanders or melioidosis.

Chemoprophylaxis

Data are currently lacking on the efficacy of postexposure chemoprophylaxis for either *B mallei* or *B pseudomallei* in humans. A recent publication noted that 13 laboratory workers, identified as having high-risk exposure to *B pseudomallei* from sniffing of 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), were given postexposure prophylaxis with a 2-week course of TMP-SMZ.⁸⁸ None of the 13 individuals developed illness or antibodies to *B pseudomallei* over the following 6 weeks; however, this response may reflect the low risk of laboratory-acquired illness from the organism as opposed to the effectiveness of antibiotic prophylaxis.^{89,90} Chemoprophylaxis recommendations are based on animal studies and in-vitro data.

Animal studies with *B pseudomallei*. Postexposure prophylaxis with 10 days of quinolones or TMP-SMZ, when given within 3 hours of subcutaneous exposure to 10^5 organisms of *B pseudomallei*, was found to be completely effective for preventing disease in white rats (verified by autopsy at 2 months postexposure).⁹¹ Another study demonstrated protection of hamsters with both doxycycline and ciprofloxacin (administered twice daily for 5 or 10 days duration) if started 48 hours before or immediately after intraperitoneal challenge with *B pseudomallei*, but relapses occurred in a few animals within 4 weeks after discontinuation of antibiotics.⁹² However, delay of antibiotic prophylaxis initiation to 24 hours after the exposure provided minimal protection, resulting only in a delay of infection that occurred 5 weeks or later after the discontinuation of antibiotics.⁹² The differences in results between the two animal models may be related to the higher susceptibility of hamsters to melioidosis.

Animal studies with *B mallei*. Doxycycline or ciprofloxacin for 5 days initiated 48 hours before or immediately after intraperitoneal challenge with 2.9×10^7 colony-forming units of *B mallei* had a protective effect in hamsters.⁹² However, the effect was temporary in some animals, with disease occurring after discontinuing the antibiotics. Relapses were associated with ciprofloxacin beginning at day 18 and with doxycycline beginning at day 28 after challenge. Necropsies of fatalities revealed splenomegaly with splenic abscesses from *B mallei*, and necropsies of the surviving animals revealed splenomegaly with an occasional abscess.⁹² However, hamsters are highly susceptible to infection from *B mallei*, and the protective effect of chemoprophylaxis in humans may be greater. Delay of ciprofloxacin or doxycycline prophylaxis initiation to 24 hours after the exposure resulted in a delay of disease, with relapses occurring in hamsters within 4 weeks of the challenge.

In-vitro susceptibility tests. Both *B pseudomallei* and *B mallei* have demonstrated sensitivity on in-vitro susceptibility testing to TMP-SMZ, tetracyclines, and augmentin, with *B mallei* also sensitive to rifampin, quinolones, and macrolides (only a few *B mallei* quinolone-resistant strains are known).^{86,93,94} *B pseudomallei* is resistant to ciprofloxacin on in-vitro testing, with MICs exceeding achievable serum drug levels.^{95,96}

Ciprofloxacin may achieve intracellular concentrations 4 to 12 times greater than that achieved in the serum, and it has been successful in treating some patients with melioidosis in spite of reported in-vitro resistance.^{97,98} Most isolates of *B pseudomallei* are resistant to rifampin,⁹⁶ and 20% of isolates in Thailand are now resistant to TMP-SMZ.

Chemoprophylaxis recommendations. Recommendations for postexposure prophylaxis are based on in-vitro and animal data, with limited or no supportive data in humans. Drugs that may be considered for chemoprophylaxis for melioidosis may include doxycycline (100 mg twice daily), tetracycline (500 mg four times daily), TMP-SMZ (one double-strength tablet twice daily), or ciprofloxacin (500 mg twice daily). For glanders, chemoprophylaxis may consist of doxycycline (100 mg twice daily), TMP-SMZ (one double-strength tablet twice daily), augmentin 500/125 (one tablet twice daily), or possibly ciprofloxacin (500 mg twice daily). The duration of treatment should be at least 14 days, but a 21-day course of therapy may be considered, based on relapses occurring in animals receiving antibiotics for 5 to 10 days following exposure. Treatment of disease requires two drugs; it is not known if a chemoprophylaxis regimen of two drugs will reduce the risk of relapse. Postexposure prophylaxis with TMP-SMZ for 21 days was given to 16 of 17 laboratory workers who had manipulated cultures of *B pseudomallei* (77% were assessed as high-risk exposures), and no individuals developed subsequent disease or seroconversion.⁹⁹ Chemoprophylaxis regimens should be adjusted based on results of sensitivity testing. Individuals who start prophylaxis, particularly if more than 24 hours after exposure, must be carefully monitored after completion of antibiotic therapy because delayed chemoprophylaxis in animal studies failed to provide protection; it only delayed the onset of symptoms.

Brucellosis

Brucellosis is a zoonotic disease caused by infection with one of six species of *Brucellae*, a group of intracellular, gram-negative coccobacilli.¹⁰⁰ The natural reservoirs for this organism are sheep, cattle, and goats. Infection is transmitted to humans by direct contact with infected animals or their carcasses, or from ingestion of unpasteurized milk or milk products. Brucellosis is not transmissible person to person. *Brucella* are highly infectious by aerosol and are still one of the most common causes of laboratory-acquired exposure,^{12,101} with an infective dose of only 10 to 100 organisms.¹⁰⁰ Symptoms generally occur within 7 to 21 days of exposure, but may occur as late as 8 weeks or longer postexposure.

Vaccination

Live animal vaccines have eliminated brucellosis in most domestic animal herds in the United States, but no licensed human vaccine is available.

Chemoprophylaxis

No FDA-approved chemoprophylaxis exists for brucellosis. A 6-week course of both rifampin (600 mg orally once daily) and doxycycline (100 mg twice daily) has been effective in the treatment of brucellosis, with relapse rates less than 5% to 10%.^{102,103} Although a 3- to 6-week course of rifampin and doxycycline may be considered as chemoprophylaxis in high-risk exposures, there are no animal or human data to support this regimen other than its effectiveness in brucellosis treatment. However, one study reported prophylaxis using doxycycline (200 mg daily) and rifampin (600 mg daily) administered to nine asymptomatic laboratory workers who seroconverted after exposure to *B abortus* serotype 1 atypical strain (a strain with low virulence).¹⁰⁴ These individuals subsequently developed symptoms of fever, headache, and chills that lasted a few days. This was in contrast to three persons who did not receive prophylaxis and had symptoms of fever, headache, and chills for 2 to 3 weeks, in addition to symptoms of 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.¹⁰⁵ Five individuals were given postexposure prophylaxis for 3 weeks (four individuals received doxycycline 100 mg twice daily plus rifampin 600 mg daily, and one pregnant laboratory worker received TMP-SMZ 160 mg/800 mg twice daily). One individual declined prophylaxis and subsequently developed brucellosis (confirmed by culture). The five individuals who received postexposure prophylaxis remained healthy and did not seroconvert.

Other combinations of drugs that may be considered for chemoprophylaxis are TMP-SMZ with doxycycline (if the patient cannot take rifampin) and ofloxacin with rifampin (if the patient cannot take doxycycline).^{106,107} Quinolones have been demonstrated to have in-vitro activity, but clinical experience with quinolones is limited, and initial experience suggests they may not be as effective as the other drugs.^{104,108}

Q Fever

Q fever is a zoonotic disease caused by a rickettsia, *Coxiella burnetii*. The natural reservoirs for this organism are sheep, cattle, and goats.^{109,110} Humans acquire Q fever infection by inhaling aerosols contaminated with the organisms, with infections resulting from as few as 1 to 10 organisms.¹⁰⁰ Q fever is not transmissible person to person. The incubation period is generally between 15 and 26 days, but has been reported to be as long as 40 days with exposures to low numbers of organisms.¹¹¹ Although this agent is deemed a category B biological warfare agent because it cannot cause massive fatalities, its low infective dose, the significant complications resulting from chronic infection (endocarditis), and its known environment stability (it may remain viable in the soil for weeks) make *C burnetii* a potential biowarfare agent.

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, the 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.¹¹² No FDA-approved vaccine is currently available for vaccination against Q fever in the United States. However, a vaccine approved in Australia (Q-Vax, manufactured by CSL Ltd, Parkville, Victoria, Australia) has been demonstrated to be safe and effective for preventing Q fever, and a similar IND vaccine (NDBR 105) has been used in at-risk researchers at Fort Detrick since 1965. The latter vaccine is available only at USAMRIID on an investigational basis.

Q-Vax. Q fever can be prevented by vaccination. The Q-Vax vaccine, currently licensed in Australia, was demonstrated to be protective in abattoir workers in Australia. Q-Vax is a formalin-inactivated, highly purified *C burnetii* whole-cell vaccine derived from the Henzerling strain, phase I antigenic state.^{113,114} Over 4,000 abattoir workers were vaccinated subcutaneously with 0.5 mL (30 μ g) 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 of vaccination (before vaccine-induced immunity) versus 97 cases in unvaccinated persons (approximately 2,200 unvaccinated individuals but the exact number is not known).¹¹³

The protective effect of the vaccine has been virtually 100%, with only two cases of Q fever occurring in 2,555 vaccinated abattoir workers between 1985 and 1990, with both cases occurring within a few days of vaccination (before immunity developed).¹¹⁵ Over 32,000 Australian abattoir workers have been vaccinated since 1981, reducing the incidence of Q fever in this high-risk group to virtually zero. Skin test postvaccination was not a useful indicator of immunogenicity, with only 31 of 52 vaccinees (60%) converting to skin test positive.¹¹⁶ However, conversion from a negative to a positive lymphoproliferative response (indicating cell-mediated immunity) was observed in 11 of 13 subjects (85%) in this same study, occurring between days 9 to 13 postvaccination.¹¹⁶ The main adverse event noted with this vaccine was the risk of severe necrosis at the vaccine site in vaccinees who had prior exposure to Q fever.^{113,117} Therefore, a skin test with 0.02 mg of the vaccine is required before vaccination. The exclusion from vaccination of individuals who tested positive on the skin test (denoting previous exposure to *C burnetii*) has eliminated sterile abscesses (Figure 21-3).^{118,119}

NDBR 105 Q fever vaccine. The NDBR 105 (IND 610) Q fever vaccine is an inactivated, lyophilized vaccine that has a preparation similar to Q-Vax. The vaccine originates from chick fibroblast cultures derived from specific pathogen-free eggs infected with the phase I Henzerling strain.

The NDBR 105 Q fever vaccine was demonstrated to be effective in animal studies.^{118,120,121} The vaccine also prevented further cases of Q fever in at-risk laboratory



Fig. 21-3. 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 Dr Herbert Thompson, MD, MPH.

workers in the Fort Detrick offensive biological warfare program during the final 4 years of the program (1965–1969), compared to an average of three cases per year before the vaccine availability.^{12,122} There has been only one case of Q fever (mild febrile illness with serologic confirmation) with use of the vaccine in the 35 years of the subsequent biodefense research program at Fort Detrick, attributed to a high-dose exposure from a breach in the filter of a biosafety cabinet.¹²³ The vaccine may have ameliorated symptoms of disease in this individual.

Skin testing is required before vaccination to identify persons with prior exposure to Q fever, performed by injecting 0.1 mL of skin-test antigen (1:1500 dilution of the vaccine with sterile water) intradermally in the forearm. A positive skin test is defined as erythema of 30 mm (or greater) or induration of 20 mm (or greater) at day 1 or later after the skin test, or erythema and induration of 5 mm (or greater) on day 7 after the test. These persons are considered to be naturally immune and do not require vaccination. Because of the risk of severe necrosis at the vaccine site, vaccination with Q fever is contraindicated in persons with a positive skin test.

The vaccine is administered by injecting 0.5 mL subcutaneously in the upper outer aspect of the arm, and is given only once. Protection against Q fever is primarily cell-mediated immunity. Markers to determine vaccine immunity to the NDBR 105 vaccine have been studied (ie, cell-mediated immunity studies, skin testing, and antibody studies pre- and postimmunization), but reliable markers have not yet been identified for the NDBR 105 vaccine. After vaccination with Q-Vax (similar to the NDBR 105 Q fever vaccine), skin test seroconversion occurred in only 31 of 52 persons (60%),^{113,116,119,124,125} but lymphoproliferative responses to *C burnetii* antigens were demonstrated to persist for at least 5 years in 85% to 95% of vaccinated persons.^{113,124} Vaccine breakthroughs have been rare in vaccinated persons.

Adverse events from the NDBR 105 vaccine were reported by 72 of 420 skin-test–negative vaccinees (17%) and were mainly local reactions, including erythema, induration, or 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 characterized by headache, chills, malaise, fatigue, myalgia, and arthralgia.¹²⁶

Other vaccines. The Soviet Union studied a live vaccine with an avirulent variant of Grita strain (M-44). Vaccinating guinea pigs with the M-44 attenuated

vaccine was associated with both persistence of the organism and mild lesions in the heart, spleen, and liver.¹²⁷ Because of the risk of endocarditis in persons with valvular heart disease, this vaccine or the pursuit of development of other attenuated vaccines for human use has not been considered safe.¹²⁷⁻¹²⁹

Current vaccine research has concentrated on efforts to develop a vaccine that induces protective immunity but allows for administration without screening for prior immunity. Partially purified subunit protein vaccines have demonstrated protection in mice and guinea pigs.¹³⁰⁻¹³² However, the proteins of these two vaccines were not cloned or well characterized to identify a single protective protein. Although DNA vaccines have been associated with strong cell-mediated immune responses, development of a DNA vaccine against Q fever is difficult because no protective antigen has been identified.¹³⁰

Vaccination is the mainstay of medical countermeasures against viral agents of bioterrorism. Both FDA-approved vaccines (eg, smallpox, yellow fever) and investigational vaccines (eg, Rift Valley fever vaccines and Venezuelan, eastern, and western equine encephalitis viruses) 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.

Alphaviruses

Venezuelan, eastern, and western equine encephalitis (VEE, EEE, and WEE) viruses are ribonucleic acid viruses of the family *Togaviridae*. Infections from these encephalitic viruses may manifest with fever, chills, headache, myalgias, vomiting, and encephalitis. Infections are naturally acquired through the bite of infected mosquitoes, but infections may also be acquired from aerosolized virus (such as in a bioterrorism event).

Vaccination

Licensed vaccinations are available for equines, but the only vaccines available for humans against VEE, EEE, and WEE are investigational. 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 for both EEE and WEE viruses are also available on an IND basis at USAMRIID. These vaccines have demonstrated efficacy in animal models and have been used in at-

Chemoprophylaxis

Prophylaxis with oxytetracycline (in a 3-g loading dose followed by 0.75 g every 6 hr) for 5 to 6 days was demonstrated to be effective for preventing disease in humans, if started 8 to 12 days after exposure.¹¹¹ Initiation of prophylaxis earlier than 7 days postexposure may only delay the onset of symptoms. Four of five men given oxytetracycline (for 5 to 6 days) within 24 hours after exposure to a small quantity of *C burnetii* only delayed disease for 8 to 10 days longer than seen in the control group who were not given chemoprophylaxis, with disease occurring approximately 3 weeks after discontinuation of therapy.¹¹¹ Based on these studies, doxycycline (100 mg orally twice daily) or tetracycline (500 mg 4 times daily for 7 days) beginning 8 to 12 days after the exposure may be considered for postexposure chemoprophylaxis to *C burnetii*.

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risk laboratory workers at the institute for more than 30 years. Because of their investigational status and limited supply, use of these vaccines in a bioterrorism event would be extremely limited.

The Venezuelan equine encephalitis TC-83 vaccine. Laboratory infections with VEE became problematic soon after the discovery of the agent in 1938. In 1943 eight cases of occupationally acquired VEE were reported.¹³³ Attempts to produce an effective and safe vaccine against VEE in the 1950s at Fort Detrick failed. As a result of live virus remaining in a poorly inactivated vaccine preparation, 14 cases of clinical illness and eight virus isolations occurred in 327 subjects who had received 1,174 vaccinations.¹³⁴

Live attenuated VEE TC-83 vaccine (IND 142, NDBR 102) was manufactured at the National Drug Company in Swiftwater, Pennsylvania, in 1965 using serial propagation of the Trinidad strain (subtype I-AB) of VEE 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 VEE was used as seed stock to propagate in the 81st passage in fetal guinea pig heart cells.¹³⁵

The TC-83 designation refers to the 83 passages in cell culture. The seed stock (81-2-4) was provided by Fort Detrick and diluted in a 1:100 ratio. 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 in a ratio of 1:400 with modified Earle's medium and 0.5% human serum albumin, 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.

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 (intraperitoneally) dose of the vaccine (containing approximately 10^6 virions). All animals survived. Additional rabbit, suckling mouse, mouse virulence, and monkey neurovirulence testing was conducted. The vaccine was protective against both subcutaneous and aerosol challenge in mice and hamsters. There was inconsistent protection in the monkey model after aerosol exposure. Postrelease potency analyses have been performed periodically over the past 35 years, showing that infectivity for all lots seems to have declined by one to two logs from the original data in the IND 142 submitted in 1965.¹³⁶

At-risk laboratory workers at Fort Detrick have received the TC-83 vaccine since 1963. VEE TC-83 lot 4-3 vaccination of at-risk USAMRIID laboratory workers from 2002 to 2005 was associated with an acceptable postvaccination 80% plaque reduction neutralization titer (PRNT₈₀) of 1:20 or greater in 136 of 169 individuals (80%). Because the vaccine is derived from epizootic strains, the vaccine may not protect against enzootic strains of VEE (subtypes II through VI) and may not adequately protect against distantly related VEE subtype I-AB variants.¹²³

The components of the TC-83 vaccine include 0.5% human serum albumin and 50 $\mu\text{g}/\text{mL}$ each of neomycin and streptomycin. The vaccine is administered as a 0.5-mL subcutaneous injection (approximately 10^4 plaque-forming units per dose) in the deltoid area of the arm.

TC-83 vaccine adverse events. The severity and frequency of adverse events from the VEE TC-83 vaccine varied with the vaccine lot. Of all lot 4-2 VEE TC-83 vaccine recipients, 40% developed mild-to-moderate systemic reactions, primarily fever, fatigue, neck pain, upper back pain, sore throat, headache, muscle ache, nausea, vomiting, and loss of appetite. In another 5% of vaccine recipients, these symptoms were severe enough to require bed rest or time off from work. The onset of these symptoms was usually abrupt. The fever lasted 24 to 48 hours, and symptoms persisted up to 3 days. The occurrence of these symptoms often had two phases, occurring initially 2 to 3 days after vaccination and recurring 7 to 18 days after vaccination. These reactions resolved without permanent effects. A change of lot of VEE TC-83 vaccine occurred in January 2002. Although the rate of mild-to-moderate reactions remained stable at 42% (32/76 vaccine recipients) with

lot 4-3, the rate of severe reactions observed was higher, occurring in 16% (12/76 subjects). No person-to-person transmission of VEE has been documented after vaccination with TC-83.¹³⁷ Local reactions are rarely seen.

The association of diabetes mellitus with VEE TC-83 vaccine is uncertain. Three cases of diabetes have been recognized after receipt of the vaccine at USAMRIID, occurring in two individuals with a strong family history of diabetes. In a study conducted after a VEE epidemic caused by virulent Trinidad strain,¹³⁸ an increased risk of developing insulin-dependent diabetes was noted, but because the size of the observed population group was limited, statistical significance was not observed. Studies involving the induction of diabetes after VEE infection in animal models were inconclusive,¹³⁹⁻¹⁴¹ and no animal model of VEE virus induction of acute, insulin-dependent diabetes exists. However, 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.¹⁴² There are many animal models in which this kind of event can be reproduced. Rhesus monkey fetuses were inoculated with VEE vaccine virus by direct intracerebral route at approximately 100 days gestation. 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.¹⁴³ VEE vaccine virus is teratogenic for nonhuman primates and must be considered a potential teratogen of humans. The wild-type VEE virus is known to cause fetal malformations, abortions, and stillbirths.¹⁴⁴

The Venezuelan equine encephalitis C-84 vaccine. The VEE C-84 formalin inactivated vaccine (IND 914, TSI-GSD 205) is made from the TC-83 production seed and 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 freeze-dried.

The VEE C-84 vaccine was protective against subcutaneous challenge but not against aerosol challenge in hamsters or cynomolgus monkeys, and protection against aerosol challenge in BALB/c mice was short-lived (less than 6 months).¹⁴⁵⁻¹⁴⁹ VEE-specific IgA was detected less frequently in mice vaccinated with the inactivated VEE C-84 vaccine than with the live attenuated VEE TC-83 vaccine. This was noted particularly in the bronchial and nasal washings, suggesting that VEE-specific IgA in the mucosal secretions may be important in protection against aerosolized VEE virus.

Therefore, the C-84 vaccine has not been used for primary vaccination against VEE, but it has been used in at-risk laboratory workers at Fort Detrick as a booster for those individuals who had received the VEE TC-83 vaccine and had either (a) an inadequate initial response with a PRNT₈₀ of less than or equal to 1:20 or (b) had an adequate response to the VEE TC-83, but PRNT₈₀ levels subsequently dropped below 1:20. The inactivated VEE C-84 vaccine demonstrated immunogenicity, with a positive response (PRNT₈₀ ≥ 1:20) following a booster dose with the vaccine observed in 87% (N=581) of individuals receiving the vaccine (1987–2001).

The components of the VEE C-84 vaccine are neomycin and streptomycin at a concentration of 50 µg/mL, sodium bisulfite, chicken eggs, and formalin. The vaccine is administered as a 0.5-mL subcutaneous injection above the triceps area. The current protocol allows for a maximum of four doses a year if postvaccination titers are not adequate. From 2002 to 2006 at USAMRIID, 8% to 33% of individuals receiving C-84 as a booster have 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 resolved without sequelae.

The western equine encephalitis vaccine. The inactivated western equine encephalitis vaccine (IND 2013, TSI-GSD 210) is a lyophilized product originating from the supernatant harvested from primary chicken fibroblast cell cultures.¹⁵⁰ The vaccine was prepared from specific pathogen-free eggs infected with the attenuated CM4884 strain of WEE virus. 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 inactivated WEE vaccine was originally manufactured by the National Drug Company. The current product, lot 2-1-91, was manufactured at the Salk Institute, Government Services Division (Swiftwater, Pa) in 1991. 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 the vaccine to be effective against intracerebral challenge with WEE in 19 of 20 mice (95%).¹⁵¹ Hamsters were protected against intraperitoneal challenge with WEE when vaccinated intraperitoneally at days 0 and 7.¹⁵² Vaccination of

horses at days 0 and 21 resulted in protection in all 17 animals against intradermal challenge at 12 months after vaccination, even in the absence of detectable WEE protective neutralizing antibodies.¹⁵³ This suggests that the vaccine may also provide protection in the absence of detectable antibody levels.

Human subjects administered 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.¹⁵⁰ 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 levels 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.

The inactivated WEE vaccine has been administered to at-risk personnel at Fort Detrick since the 1970s. Pittman et al evaluated the vaccine for its immunogenicity and safety in 363 at-risk workers enrolled in evaluation trials at USAMRIID between 1987 and 1997.¹⁵⁴ All volunteers were injected subcutaneously 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 PRNT₈₀ fell below 1:40, a booster dose (0.5 mL) was given 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 PRNT₈₀ of greater than or equal to 1:40. Seventy-six of 115 initial nonresponders (66%) were 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.

Passive collection of local and systemic adverse events from the inactivated WEE vaccine was the method used from 1987 to 1997. Of the 363 vaccinees who received three initial injections, only five 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 Special Immunizations Clinic at USAMRIID 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, the vaccination of pregnant women is not advisable.

The eastern equine encephalitis vaccine. The formalin-inactivated EEE vaccine (TSI-GSD 104) was manufactured in 1989 by the Salk Institute.¹⁵⁵ The seed for the EEE virus was passaged twice in adult mice, twice in guinea pigs, and nine times in embryonated eggs.¹⁵⁶ The final EEE vaccine was derived from supernatant fluids bearing virus accumulated from three successive passages on primary chick embryo fibroblast cell cultures prepared from specific pathogen-free eggs infected with the attenuated PI-6 strain of virus. The supernatant was harvested and filtered, and the virus then inactivated with formalin. The product was then lyophilized for storage at -20°C .

The EEE vaccine contains 50 $\mu\text{g}/\text{mL}$ 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 PRNT₈₀ of 1:40 or greater is considered adequate. Should the titer fall 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.

Animal studies demonstrated that the EEE vaccine is 95% protective against intracerebral challenge in guinea pigs, with survival correlating to serum neutralizing antibody titers.¹⁵⁷ Vaccination of horses was also protective against intradermal challenge at 12 months postvaccination, even with absence of detectable neutralizing antibody titers in 16 of the 17 animals, suggesting the vaccine may also provide protection in this species in the absence of detectable antibody levels.¹⁵³ The vaccine has been given to at-risk laboratory workers at Fort Detrick for over 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 PRNT₈₀ of 1:40 or greater. Intradermal vaccination with EEE resulted in an adequate titer in 66% of the initial nonresponders.

Adverse events from the EEE vaccine occurred in approximately 20% individuals, consisting 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 vaccine site have also been reported.

Postexposure Prophylaxis

No treatment has been shown to alter the course of VEE, WEE, or EEE disease in humans once disease has been contracted. The treatment is limited to supportive care; no currently known antiviral drug is effective.

New Vaccine Research

The live attenuated VEE vaccine candidate V3526 was scheduled to replace the 40-year-old VEE TC-83 IND vaccine. The newer-generation VEE vaccine candidate had improved activity against VEE enzootic strains. However, because of high rates of severe neurologic adverse events in clinical phase I trials, further development of this product was halted. This was unexpected with the new V3526 vaccine candidate because it demonstrated less reactogenicity in nonhuman primate studies than the VEE TC-83 product. Recently, the V3526 vaccine candidate was inactivated and transferred to the National Institute of Allergy and Infectious Diseases for future preclinical and clinical development as a multidose primary series. Many of the existing equine encephalitis vaccines have been under IND status for over 30 years, yet because of funding shortfalls, these products have never been transitioned from development to licensure.

Smallpox

Smallpox is caused by variola virus, of the genus *Orthopoxvirus*. Smallpox is recognized to have occurred in ancient Egypt, China, and India, and for centuries was the greatest infectious cause of human mortality. The disease was declared eradicated in 1980, after an intensive vaccination program. Subsequently, all known stocks of variola virus were destroyed, with the exception of stock at two World Health Organization collaborating centers, the CDC, and the Russian State Research Center of Virology and Biotechnology. Smallpox has been designated a category A biothreat agent because of its high mortality, high transmissibility, and past history of massive weaponization by the former Soviet Union.

Vaccination

History of smallpox vaccination. Vaccination with smallpox was recorded in 1,000 BCE in India and China, where individuals were inoculated with scabs or pus from smallpox victims (either in the skin or nasal mucosa), producing disease that was milder than naturally occurring smallpox. In the 18th century in Europe, scratching and inoculation of the skin with

pock material, known as variolation, was performed, resulting in a 90% reduction in mortality and long-lasting immunity. Variolation performed in Boston in 1752 resulted in a smallpox death rate of 1% (2,124 persons) compared to a death rate of 10% in unvaccinated persons (5,545 persons).

In 1796 Edward Jenner noticed that milkmaids rarely had smallpox scars, and subsequently discovered that inoculation of the skin with cowpox taken from a milkmaid's hand resulted in immunity. In 1845 the smallpox vaccine was manufactured in calfskin. Production of the vaccine became regulated in 1925, with use of the New York City Board of Health strain of vaccinia as the primary US vaccine strain. Vaccination eventually led to eradication of the disease, with the last known case of naturally occurring smallpox reported in 1977. 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. Because of the recent risk of bioterrorism, vaccination of smallpox in at-risk military personnel was resumed in 2003.

The smallpox vaccine. Dryvax, the smallpox vaccine, manufactured by Wyeth Laboratories (Marietta, Pa), is a live-virus preparation of vaccinia virus made from calf lymph. The calf lymph is purified, concentrated, and lyophilized. The diluent for the vaccine contains 50% glycerin and 0.25% phenol in US Pharmacopeia sterile water, with no more than 200 viable bacterial organisms per mL in the reconstituted product. Polymyxin B sulfate, dihydrostreptomycin sulfate, chlortetracycline hydrochloride, and neomycin sulfate are added during the processing of the vaccine, and small amounts of these antibiotics may be present in the final product. The reconstituted vaccine contains approximately 100 million infectious vaccinia viruses per mL, and it is intended only for administration into the superficial layers of the skin by multiple puncture technique.

The vaccine is administered by scarification with a bifurcated needle, by applying three punctures to scarify the epidermis on the upper arm for primary vaccination, and 15 punctures for booster vaccinations. The individual is followed after vaccination to document a take, which indicates immunity against smallpox. Six to 8 days after the primary vaccination, a primary major reaction to the vaccine develops, with a clear vesicle or pustule of approximately 1 cm diameter. The site then scabs over by the end of the second week, with the scab drying and separating by day 21 to 28 (Figure 21-4). In individuals with prior vaccination, an immune response is generally observed. The immune response is an accelerated response, with a pruritic papule appearing between days 1 and 3 post-

vaccination. Individuals who do not exhibit either a primary major reaction or an immune response (ie, individuals with erythema, pruritus, or induration but no papule or vesicle) require revaccination. If no primary reaction is noted after revaccination (and ensuring proper technique in vaccine administration was used), these individuals are considered immune. At some point in the future, which may be years, the immunity of these individuals may wane, and revaccination at that time may result in a take.

Smallpox vaccine has been demonstrated to be effective in prevention of smallpox. 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. Health officials recommend vaccination with confirmation of a take every 3 years for those who are likely to be exposed to the virus (ie, a smallpox outbreak). However, individuals working with variola in the laboratory are recommended to have a yearly smallpox vaccination.

Secondary attack rates 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 higher risk for acquiring infection. In an outbreak recorded in the Shekhupura District of Pakistan during the smallpox era, the secondary attack rate in vaccinated persons was only 4% in persons

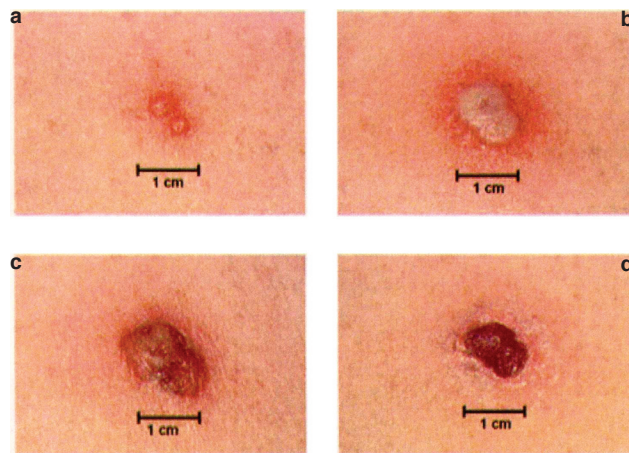


Fig. 21-4. Primary reaction to smallpox vaccination, at (a) day 4, (b) day 7, (c) day 14, and (d) day 21.

Reproduced from: Centers for Disease Control and Prevention Web site. Available at: <http://www.bt.cdc.gov/agent/smallpox/smallpox-images/vaxsit5a.htm>. Accessed March 26, 2007.

vaccinated within the previous 10 years (5/115) and 12% in persons vaccinated over 10 years before (8/65), compared to 96% in unvaccinated persons (26/27).^{158,159} 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 of only 1.4% in individuals who had received the smallpox vaccine within the previous 10 years, compared to 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 over 20 years before. Postexposure vaccination resulted in 27% less mortality when compared (retrospectively) with smallpox patients who were never vaccinated.¹⁵⁸ However, postexposure vaccination was only helpful if given within 7 days of the exposure. Postexposure vaccination is most effective if given within 3 days of exposure (preferably within 24 hours), but may still be effective if given within 7 days.¹⁶⁰

Contraindications and adverse events. Smallpox vaccination is contraindicated in the preoutbreak setting for individuals who

- have a history 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;
- are immunocompromised;
- have a serious allergy to any of the vaccine components; or
- are younger than 1 year old.¹⁶¹

The CDC has recently recommended underlying cardiac disease (history of ischemic heart disease, myocarditis, or pericarditis) or significant cardiac risk factors as relative contraindications to the vaccine. The ACIP also does not recommend vaccination of persons younger than 18 years old in the preoutbreak setting.¹⁶¹ Vaccination is also contraindicated in persons with household members who have a history of eczema or active skin conditions as described above, are immunosuppressed, or are pregnant. Although the presence of an infant in the household is not a contraindication for vaccination of the adult member, the ACIP recommends deferring vaccination of individuals with households that have infants younger than 1 year old because of data indicating a higher risk for adverse events among primary vaccinees in this age group.¹⁶¹ Because skin lesions resulting from the varicella vaccine may be confused with vaccinia lesions, simultaneous administration of the smallpox

and varicella vaccine is not recommended. However, in an outbreak situation, there are no contraindications to vaccination for any person who has been exposed to smallpox (Tables 21-3 and 21-4).

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 21-5 and 21-6).^{162,163}

Vaccinia can be transmitted from a vaccinee's unhealed vaccination site to other persons by close contact and can lead to the same adverse events as intentional vaccination (Figure 21-5). Incidence of transmission to contacts during the most recent military vaccination experience was 47 per million vaccinees. Additionally, vaccinees may inoculate themselves and cause infection in areas such as the eye, which is associated with significant morbidity (Figure 21-6). Incidence of inadvertent self-inoculation in the military was 107 per million vaccinees.¹⁶² 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.

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 21-6).¹⁶⁴ Topical treatment with trifluridine (Viroptic; Catalyca Pharmaceuticals, Inc, Greenville, NC) or vidarabine (Vira-A) is often recommended, although treatment of ocular vaccinia with either of these drugs is not specifically approved by the FDA.¹⁶⁵ Most published experience is with use of vidarabine, but this drug is no longer manufactured. Vaccinia immune globulin (VIG) may be recommended in severe cases of ocular vaccinia, but it is contraindicated in individuals with vaccinal keratitis because of the risk of corneal clouding. Corneal clouding was observed in 4 of 22 persons with vaccinal keratitis who received VIG.¹⁶⁶ A subsequent study in rabbits showed that treatment of vaccinal keratitis with VIG was associated with both corneal scarring and persistent and larger satellite lesions compared to control animals.¹⁶⁷

Generalized vaccinia is characterized by a disseminated maculopapular or vesicular rash, frequently on an erythematous base and typically occurring 6 to 9

TABLE 21-3

CONTRAINDICATIONS TO SMALLPOX VACCINATION (PRE-EVENT VACCINATION PROGRAM)*

Condition	Contraindication
<p>Allergies to vaccine components</p> <p><i>Each Dryvax (Wyeth Laboratories; Marietta, Pa) vaccine lot contains antibiotics and preservatives. Specific allergies to these products may occur. Appropriate history of such allergies should be obtained and may negate vaccine administration when smallpox is not present.</i></p> <p>Current Dryvax contains following antibiotics:</p> <ul style="list-style-type: none"> • polymyxin B sulfate • streptomycin sulfate • chlortetracycline hydrochloride • neomycin sulfate 	<p>If smallpox is present and the risk of contact is great, the vaccine should be administered with subsequent use of an appropriate antihistamine or other medication.</p>
<p>Pregnancy</p>	<p>Do not administer if pregnant and advise vaccinee not to become pregnant for 1 month after vaccination.</p>
<p>Infancy</p>	<p>Younger than 1 year old</p>
<p>Immunodeficiency</p>	<p>Includes any disease with immunodeficiency (congenital or acquired) as a component:</p> <ul style="list-style-type: none"> • HIV infection • AIDS • Many cancers
<p>Immunosuppressive therapy</p> <p><i>Immunosuppression from some medications may last for up to 3 months after discontinuation</i></p>	<ul style="list-style-type: none"> • 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 or longer)
<p>Eczema or atopic dermatitis or Darier's disease (keratosis follicularis)</p>	<p>History or presence of eczema or atopic dermatitis or Darier's disease. (Even patients with "healed" eczema or atopic dermatitis may manifest complications. They should not be vaccinated, and they should avoid contact with a recent vaccinee.)</p>
<p>Skin disorders</p> <p><i>The size and extent of the non-eczema/atopic skin disorder may be sufficiently small that vaccination can be safely performed. However, all such patients must be counseled to take great care to avoid any transfer from the primary site to the affected skin. Persons with conditions or injuries that cause extensive breaks in the skin should not be vaccinated until the condition resolves.</i></p>	<p>Disruptive or eruptive conditions:</p> <ul style="list-style-type: none"> • Severe acne • Burns • Impetigo • Contact dermatitis or psoriasis • Chickenpox
<p>Cardiovascular disease</p>	<p>Reports of myopericarditis and cardiovascular disease have resulted in recent exclusion of individuals with history of these disorders.</p>

* Vaccine contraindicated if listed condition exists either in the potential vaccinee, or if condition exists in household contact or other close physical contact of the vaccinee (excluding history of vaccine allergy or known cardiovascular disease in contacts). During a smallpox outbreak, the risk of vaccination must be weighed against the risk of disease. (During the smallpox era, there was no absolute contraindication to vaccination.)

HIV: human immunodeficiency virus

AIDS: acquired immunodeficiency syndrome

Adapted from: Centers for Disease Control and Prevention. Smallpox vaccination and adverse events training module. 2002. Available at: <http://www.bt.cdc.gov/training/smallpoxvaccine/reactions/contraindications.html>. Accessed March 23, 2007.

TABLE 21-4
PRECAUTIONS FOR SMALLPOX VACCINATION
(PRE-EVENT VACCINATION PROGRAM)

Condition	Precaution
Active eye disease of the conjunctiva or cornea	Persons with inflammatory eye diseases may be at increased risk for inadvertent inoculation due to touching or rubbing of the eye.
Inflammatory eye disease requiring steroid treatment	The Advisory Committee for Immunization Practices recommends that persons with inflammatory eye diseases requiring steroid treatment defer vaccination until the condition resolves and the course of therapy is complete.
Moderately or severely ill at the time of vaccination	Ill persons should usually not be vaccinated until recovery.
Breastfeeding	Whether the virus transmitted in breast milk is unknown. Close contact may also increase chance of transmission to infant. The product label of the smallpox vaccine recommends individuals not breastfeed after vaccination (Dryvax [Package insert]. Marietta, Pa: Wyeth Laboratories, 1994)

Adapted from: Centers for Disease Control and Prevention. Smallpox vaccination and adverse events training module. 2002. Available at: <http://www.bt.cdc.gov/training/smallpoxvaccine/reactions/contraindications.html>. Accessed March 23, 2007.

days after primary vaccination (Figure 21-7). Lane reported 242.5 cases per million primary vaccinations and 9.0 cases per million revaccinations in a 1968 10-state survey of smallpox vaccination complications.¹⁶⁸ The rash usually resolves without therapy. Treatment with VIG is restricted to those who are systemically ill or have an immunocompromising condition. Contact precautions should be used to prevent further transmission and nosocomial infection. Generalized vaccinia must be distinguished from other postvaccination exanthems, such as erythema multiforme and roseola vaccinatum (Figure 21-8).

Eczema vaccinatum may occur in individuals with a history of atopic dermatitis, regardless of current disease activity, and can be a papular, vesicular, or pustular rash (Figures 21-9 and 21-10). Historically, eczema vaccinatum occurred at a rate of 14.1 and 3.0 per million primary and revaccinations, respectively¹⁶⁸; however, in more recent military experience, there were no cases of eczema vaccinatum in 450,293 smallpox vaccinations (of which 70.5% were primary vaccinations).¹⁶³ The rash may be generalized or localized with involvement anywhere on the body, with a predilection for areas of previous atopic dermatitis lesions. Individuals with eczema vaccinatum are generally systemically ill and require immediate therapy with VIG. The mortality rate of individuals with eczema vaccinatum was 7% (9/132), even with VIG therapy. A measurable antibody response developed in 55 of the 56 survivors who had antibody titers obtained after VIG administration.¹⁶⁹ No antibody response was detected in five persons with fatal eczema vaccinatum cases who had post-VIG antibody titers measured.

TABLE 21-5
ADVERSE EVENTS AFTER SMALLPOX VACCINATION

Event Type	US Department of Defense Rate per Million Vaccinees* (95% confidence interval)	US Civilian Historical Rate per Million Vaccinees
Generalized vaccinia, mild	80 (63–100)	45–212 [†]
Inadvertent self-inoculation	107 (88–129) [§]	606 [†]
Vaccinia transfer to contact	47 (35–63)	8–27 [†]
Encephalitis	2.2 (0.6–7.2)	2.6–8.7 [†]
Acute myopericarditis	82 (65–102)	100 [‡]
Eczema vaccinatum	0 (0–3.7)	2–35 [†]
Progressive vaccinia	0 (0–3.7)	1–7 [†]
Death	0 (0–3.7)	1–2 [†]

* US military vaccinations from December 13, 2002, through May 28, 2003.

[†] Based on adolescent and adult smallpox vaccinations from 1968 studies (both primary vaccination and revaccination).

[‡] Based on case series in Finnish military recruits vaccinated with the Finnish strain of vaccinia.

[§] Includes 38 inadvertent inoculations of the skin and 10 of the eye.

Data source: Grabenstein JD, Winckenwerder W. US military smallpox vaccination program experience. *JAMA*. 2003;289:3278–3282.

TABLE 21-6

VACCINIA IMMUNE GLOBULIN ADMINISTRATION FOR COMPLICATIONS OF SMALLPOX (VACCINIA) VACCINATION

Indicated	Not Recommended
<ul style="list-style-type: none"> • Inadvertent inoculation (only for extensive lesions or ocular implantations without evidence of vaccinia keratitis) • Eczema vaccinatum • Generalized vaccinia (only if severe or recurrent) • Progressive vaccinia 	<ul style="list-style-type: none"> • Inadvertent inoculation (mild instances) • Generalized vaccinia (mild or limited—most instances) • Erythema multiforme • Postvaccination encephalitis • Isolated vaccinia keratitis (may produce severe corneal opacities)

Adapted from: Centers for Disease Control and Prevention. Smallpox vaccination and adverse events training module. 2002. Available at: <http://www.bt.cdc.gov/training/smallpoxvaccine/reactions/contraindications.html>. Accessed March 23, 2007.



◀
Fig. 21-5. 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 Web site. Photograph: Courtesy of Allen W Mathies, MD, and John Leedom, MD, California Emergency Preparedness Office, Immunization Branch. Available at: <http://phil.CDC.gov>. Accessed June 14, 2006.



Fig. 21-6. 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 Web site. Photograph: Courtesy of Allen W Mathies, MD, and John Leedom, MD, California Emergency Preparedness Office, Immunization Branch. Available at: <http://phil.CDC.gov>. Accessed June 14, 2006.



Fig. 21-7. Generalized vaccinia. This 8-month-old infant developed a generalized vaccinia reaction after having been vaccinated. Generalized vaccinia is a widespread rash, which involves sores on parts of the body away from the vaccination site resulting from vaccinia virus traveling through the blood stream. Image 4644.

Reproduced from: Centers for Disease Control and Prevention Public Health Image Library Web site. Photograph: Courtesy of Allen W Mathies, MD, California Emergency Preparedness Office, Immunization Branch. Available at: <http://phil.CDC.gov>. Accessed June 14, 2006.



Fig. 21-8. This child displays a generalized erythematous eruption called roseola vaccinatum after receiving a primary smallpox vaccination. Eruptions such as this one are common after vaccination and, although often dramatic in appearance, these are largely benign. There is no evidence of systemic or cutaneous spread of the vaccinia virus, and live virions cannot be recovered from the involved sites. The older literature from the compulsory vaccination era used an imprecise nosology for a wide range of benign post vaccination exanthems. Terms such as generalized vaccinia and erythema multiforme that occur in the older literature must be interpreted cautiously because on retrospective analysis, it is clear that they encompassed much more than those specific entities.

Data source: Lewis FS, Norton SA, Bradshaw RD, Lapa J, Grabenstein JD. Analysis of cases reported as generalized vaccinia during the US military smallpox vaccination program, December 2002 to December 2004. *J Am Acad Dermatol.* 2006;55:23–31. (Personal communication, Colonel Scott A. Norton, MD, MPH, former Chief of Dermatology, Walter Reed Army Medical Center.) Reproduced from: Centers for Disease Control and Prevention Public Health Image Library Web site. Photograph: Courtesy of Arthur E Kaye, Centers for Disease Control and Prevention. Available at: <http://phil.CDC.gov>. Accessed June 14, 2006.



Fig. 21-9. Eczema vaccinatum. This 8-month-old boy developed eczema vaccinatum after he had acquired vaccinia from a sibling recently vaccinated for smallpox. Eczema vaccinatum is a serious complication that occurs in people with atopic dermatitis who come in contact with the vaccinia virus. These individuals are at special risk of implantation of vaccinia virus into the diseased skin. 1969. Image 3311.

Reproduced from: Centers for Disease Control and Prevention Public Health Image Library Web site. Photograph: Courtesy of Arthur E Kaye, Centers for Disease Control and Prevention. Available at: <http://phil.CDC.gov>. Accessed June 14, 2006.

Contact precautions should be used to prevent further transmission and nosocomial infection.

Progressive vaccinia is a rare, severe, and often fatal



Fig. 21-10. Eczema vaccinatum. This 28-year-old woman with eczema vaccinatum contracted it from her vaccinated child. She had a history of atopic dermatitis, and her dermatitis was inactive when her child was vaccinated. As a therapy, she was given vaccinia immune globulin, idoxuridine eye drops, and methisazone, resulting in healed lesions, no scarring, and no lasting ocular damage. Image 4621. Reproduced from: Centers for Disease Control and Prevention Public Health Image Library Web site. Photograph: Courtesy of Allen W Mathies, MD, California Emergency Preparedness Office, Immunization Branch. Available at: <http://phil.CDC.gov>. Accessed June 14, 2006.

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 21-11, 21-12, and 21-13). This condition carries a high mortality rate and should be aggressively treated with VIG, debridement, intensive monitoring, and tertiary medical center level support. Those at highest risk include persons with congenital or acquired immunodeficiencies, human immunodeficiency virus infection/acquired immunodeficiency syndrome, cancer, or autoimmune



Fig. 21-11. Progressive vaccinia. This patient with progressive vaccinia required a graft to correct the necrotic vaccination site. One of the most severe complications of smallpox vaccination, progressive vaccinia is almost always life threatening. Persons who are immunosuppressed are most susceptible to this condition. Image 4624. Reproduced from: Centers for Disease Control and Prevention Public Health Image Library Web site. Photograph: Courtesy of Allen W Mathies, MD, California Emergency Preparedness Office, Immunization Branch. Available at: <http://phil.CDC.gov>. Accessed June 14, 2006.

disease, or who have undergone organ transplantation or immunosuppressive therapy. Historical rates of progressive vaccinia ranged from 1 to 3 per million vaccinees historically,¹⁶⁸ no cases in 450,293 US military vaccinees,¹⁶³ and no cases (that met case definition) in 38,440 US civilian vaccinees in 2003.¹⁷⁰ Anecdotal experience has shown that despite treatment with VIG, individuals with cell-mediated immunity defects have a poorer prognosis than those with humoral defects. A recent animal study showed that both topical and intravenous cidofovir were effective in treating vaccinia necrosis in mice deficient in cell-mediated immunity.¹⁷¹ Topical cidofovir was more effective than intravenous cidofovir, and the administration of both cidofovir preparations was superior to either preparation alone. Infection control measures should include contact and respiratory precautions to prevent transmission and nosocomial infection.

Central nervous system disease, which includes postvaccinial encephalopathy and postvaccinial encephalomyelitis, although rare, is the most frequent cause of



Fig. 21-12. Progressive vaccinia. This patient presented with progressive vaccinia after having been vaccinated for smallpox. Progressive vaccinia is one of the most severe complications of smallpox vaccination and is almost always life threatening. Although it was rare in the past, the condition may be a greater threat today because of the larger proportion of susceptible persons in the population. Image 4592. Reproduced from: Centers for Disease Control and Prevention Public Health Image Library Web site. Photograph: Courtesy of California Department of Health Services. Available at: <http://phil.CDC.gov>. Accessed June 14, 2006.



Fig. 21-13. Progressive vaccinia after debridement. Image 4594. Reproduced from: Centers for Disease Control and Prevention. Available at: <http://phil.CDC.gov>. Accessed June 14, 2006.

death related to smallpox vaccination.¹⁶⁸ Postvaccinal 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 postvaccination and include seizures, hemiplegia, aphasia, and transient amnesia. Histopathologic findings include cerebral edema, lymphocytic meningeal inflammation, ganglion degeneration, and perivascular hemorrhage. Patients with postvaccinal encephalopathy who survive can be left with cerebral impairment and hemiplegia. Postvaccinal 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 postvaccination.^{164,172} Neff's 1963 national survey detected 12 cases of postvaccinal encephalitis among 14,014 vaccinations.¹⁷³ Symptoms progress to amnesia, confusion, disorientation, restlessness, delirium,



Fig. 21-14. Fetal vaccinia. Image 3338. Photograph: Courtesy of the Centers for Disease Control and Prevention. Available at: <http://phil.CDC.gov>. Accessed June 14, 2006.

drowsiness, and seizures. The cerebral spinal fluid has normal chemistries and cell count. 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.^{174,175}

Fetal vaccinia, which results from vaccinia transmission from mother to fetus, is a very rare but serious complication of smallpox vaccination during or immediately before pregnancy (Figure 21-14). Fewer than 40 cases have been documented in the world's literature.¹⁶²

Myopericarditis, although previously reported as a rare complication of vaccination using vaccinia strains other than the New York City Board of Health strain, was not well recognized until reported during active surveillance of the Department of Defense's 2002–2003 vaccination program (Figure 21-15).^{176,177} The mean time from vaccination to evaluation for myopericarditis was 10.4 days, with a range of 3 to 25 days. Sixty-seven symptomatic cases were reported among 540,824 vaccinees, for a rate of 1.2 per 10,000 vaccinations. Reports of myocarditis in 2003 vaccinees raised concerns about carditis and cardiac deaths in individuals undergoing smallpox vaccination. Of 36,217 vaccinees, 21 cases of myopericarditis were reported with 19 cases (90%) occurring in revaccinees. The median age of the affected vaccinees was 48 years, and there was a predominance of females. Eleven of the individuals were hospitalized, but there were no

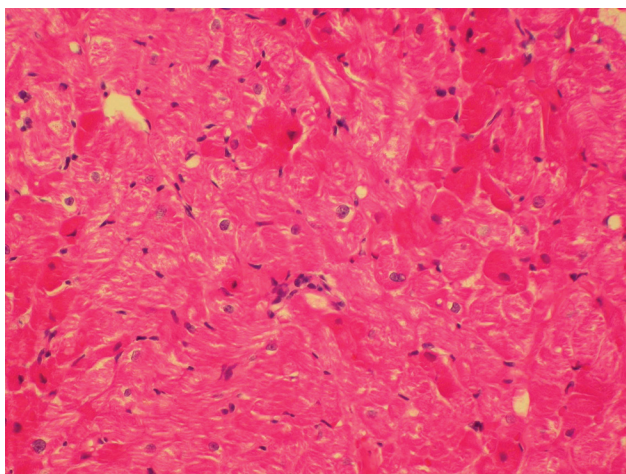


Fig. 21-15. Histopathology of vaccine-related myopericarditis showing a nonspecific lymphocytic infiltrate. Reproduced with permission of Department of Pathology, Brooke Army Medical Center, Texas.

fatalities. The military experience included 37 cases of myopericarditis of 440,293 vaccinees, for a rate of 82 per million vaccinees.¹⁶³ Additionally, ischemic cardiac events, including fatalities, have been reported following vaccination with the vaccinia vaccine (Dryvax). Although no clear association has been found, history of ischemic heart disease and the presence of significant cardiac risk pose relative contraindications for smallpox vaccination. Consequently, individuals with a history of myocarditis, pericarditis, or ischemic heart disease should not be vaccinated.^{176–178}

In a smallpox release from a bioterrorism event, individuals would be vaccinated according to the current national policy, which recommends vaccination initially of higher-risk groups: individuals directly exposed to the agent, household contacts or individuals with close contact to smallpox cases, and medical and emergency transport personnel. Ring vaccination of contacts and contact 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. 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 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.

New Vaccine Research

To develop a replacement vaccine for Dryvax and other first-generation live vaccines, researchers must produce a vaccine safe enough by current standards for widespread clinical use in a population with large segments of immunosuppressed individuals, but still induces an adequate cell-mediated immune response. Dryvax and other first-generation vaccines are 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 is preferable to the animal-derived product.^{179,180} Advances in technology and the ability to replicate vaccinia in high concentrations in a variety of cell cultures make such second-generation vaccines possible.

ACAM 2000, a candidate smallpox vaccine, is a cell-culture replicated product derived from Dryvax.^{181,182} ACAM 1000 was one of six clones of *vaccinia* obtained by serial passage and plaque picking at terminal dilution, selected for its similar immunogenicity to Dryvax in animal testing and lower neurovirulence in mice and

monkeys. The ACAM 1000 pilot production vaccine was produced in MRC-5 human diploid lung fibroblast cells. To overcome production capacity problems, the African green monkey (Vero) cell line was used after 10 passages to produce the ACAM 2000 Vero cell vaccine. Animal studies have confirmed high degrees of similarity among the ACAM 1000 master virus seed, the ACAM 2000 production vaccine, and Dryvax. Neurovirulence profiles for the ACAM 1000 and ACAM 2000 vaccine were similar, but lower than the profile for Dryvax. Phase 2 and 3 clinical trials have revealed that like Dryvax, ACAM 2000 is associated with myopericarditis. The significance of ACAM 2000's cardiac adverse effects remains to be determined.¹⁸⁰

Other approaches to developing a safe vaccine have used "non-replicating" and genetically modified "defective" viruses. Modified vaccinia ankara (MVA), a nonreplicating vaccinia virus, was produced by 574 serial passages in chicken embryo fibroblasts, resulting in a vaccinia strain safe for use in immunocompromised individuals. MVA was safely given to 150,000 persons.¹⁸³ MVA's main problem is that production in chicken embryos does not have an optimal safety profile. Production batches may consist of hundreds of eggs, which carry a risk of contamination with adventitious viruses, a problem that cannot be corrected with viral inactivation procedures. MVA can be replicated in mammalian cells, but the passage in permanent mammalian cell lines risks production of a viral strain with increased mammalian virulence. Defective vaccinia viruses have been developed by deleting a gene essential for viral replication (uracil DNA glycosylase). One such vaccine candidate, defective vaccinia virus Lister, is blocked in late gene expression from replication in any but the complementing permanent cell line. MVA and defective vaccinia virus Lister have similar safety and immunogenicity profiles.¹⁷⁹

Immunoprophylaxis

There are limited studies on the effect of VIG in conjunction with the smallpox vaccine for preventing smallpox in contact cases.¹⁸⁴⁻¹⁸⁶ A 1961 study by Kempe¹⁸⁴ 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 to 1.5% of contacts (5/326) who received both the smallpox vaccine and VIG therapy.¹⁸⁴ Research published a year later by Marennikova studied the effect of antivaccinia gamma globulin given to 13 of 42 persons who had been in close contact with smallpox patients.¹⁸⁵ 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. Thirteen of the 29 persons not given antivaccinia gamma globulin developed smallpox. However, there are no clinical trials providing evidence that giving VIG in conjunction with the smallpox vaccine as prophylaxis has a greater survival benefit than vaccination alone.^{187,188} There are currently two VIG preparations: (1) an intravenous and (2) an intramuscular formulation. The intravenous formulation recently received FDA approval and has become the formulation of first choice.¹⁸⁹ Intravenous VIG has the advantage of immediate and higher antibody levels (2.5 times the level obtained with the intramuscular VIG), and has a similar side effect profile as intramuscular VIG.¹⁸⁹ Supplies of VIG are limited and are used primarily for complications from the smallpox vaccine. VIG does not currently have a role in smallpox prevention.¹⁹⁰

Chemoprophylaxis

The acyclic nucleoside phosphonate HPMPA (or (S)-1-(3-hydroxy-2-phosphonyl-methoxypropyl) cytosine) known as cidofovir (Vistide, Gilead, Foster City, Calif) has broad-spectrum activity against DNA viruses, including the herpes viruses, papillomavirus, adenovirus, and poxviruses.¹⁹¹⁻¹⁹³ Cidofovir has a pronounced and long-lasting inhibition of viral DNA synthesis allowing for infrequent (weekly or bimonthly) dosing.¹⁹⁴ The drug has been approved by the FDA for treating cytomegalovirus retinitis in acquired immunodeficiency syndrome patients. Cidofovir has been used off-label to treat orthopox infections.

Studies of cidofovir demonstrated improved or prolonged survival in BALB/c mice and mice with severe combined immunodeficiency infected with vaccinia virus, as well as cowpox-infected mouse models, even when treatment was initiated as long as 5 days before and up to 96 hours after infection.¹⁹⁵ The greatest benefit of cidofovir prophylaxis was observed when it was administered within 24 hours before or after exposure.¹⁹⁶⁻¹⁹⁸ Nonhuman primate studies have demonstrated improved survival in monkeypox and smallpox models.¹⁹⁹ In humans, cidofovir has been found effective in the treatment of the poxvirus infection molluscum contagiosum in acquired immunodeficiency syndrome patients. However, treatment of disseminated vaccinia, smallpox, or monkeypox with cidofovir is not FDA approved. Such treatment would be off-label use based on efficacy against these viruses in animal models and anecdotal evidence of efficacy in human poxvirus (molluscum contagiosum) infections.

The animal and human data suggest that cidofovir may be effective in therapy and also in short-term

prophylaxis of smallpox, if given within 5 days of exposure. One dose of intravenous cidofovir may provide potential protection for 7 days.¹⁹⁴ Dose-related nephrotoxicity is the principal complication of cidofovir therapy in humans. Toxicity may be minimized by concomitant intravenous hydration with saline and oral probenecid.²⁰⁰ The probenecid is generally given orally as a 2-g dose 3 hours before the cidofovir infusion, and again at 2 and 8 hours after infusion. Both the Department of Defense and CDC currently have IND protocols for use of cidofovir in smallpox.

The new Siga drug, 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}, is a potent and specific inhibitor of orthopoxvirus replication. The drug is active against multiple species of orthopoxviruses, including variola virus and cidofovir resistant cowpox variants. This oral drug has been shown to be effective in preventing death in animal models of smallpox infection.²⁰¹

Viral Hemorrhagic Fevers

Countermeasures against the viruses that cause viral hemorrhagic fevers (VHFs) remain a top research priority because of the dearth of licensed vaccines and therapeutic agents to counteract these pathogens. Some success has been achieved with antiviral medications (primarily ribavirin), passive treatment using sera from previously infected donors, and vaccine development. Attempts at immunomodulation with various medications have been less successful. Pathogenesis, prevention, infection control measures, and management of patients with VHF are reviewed in other chapters specifically dedicated to VHF and infection control. This chapter will discuss potential countermeasures to VHFs most likely to be used as biological weapons (Table 21-7).

Vaccination

The only licensed US vaccine for VHFs is the 17D live attenuated yellow fever vaccine. This vaccine has substantially diminished the burden of yellow fever infection worldwide and is well tolerated, although contraindicated in immunosuppressed patients and used with caution in elderly people.²⁰² The vaccine would probably not be useful for postexposure prophylaxis because of yellow fever's short incubation time (although postexposure use of the vaccine has never been studied).²⁰³ A live attenuated vaccine against Argentine hemorrhagic fever, known as Candid 1, demonstrated efficacy in a field study among 6,500 agricultural workers in Argentina²⁰⁴: 22 patients receiving placebo devel-

oped Argentine hemorrhagic fever, compared to only 1 patient who received the Candid 1 vaccine. This vaccine is not licensed in the United States.

A number of vaccines developed and licensed in other countries may have efficacy against VHFs. Hantavax (Korea Green Cross Corporation, Yongin-si, 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 efficacy²⁰⁵; however, the humoral immune response, when measured by PRNT₈₀ antibodies, was considered protective in only 33.3% of vaccine recipients.²⁰⁶

More recent exploration into vaccine candidates for hantaviruses, such as DNA vaccines²⁰⁷ and vaccinia-vectored constructs,²⁰⁸ has suggested other potential vaccine options. An inactivated Rift Valley fever vaccine under IND status is used in the Special Immunizations Program at USAMRIID for laboratory workers who may be exposed to the virus.²⁰⁹ A live attenuated vaccine for Rift Valley fever has also been developed, and is also considered an IND, awaiting further testing. Substantial research has focused on the development of an effective Ebola vaccine. Unfortunately, demonstration of protection in murine models has not translated into successful Ebola vaccines in nonhuman primate models. Three of these unsuccessful vaccines involve (1) Venezuelan equine encephalitis virus replicon particles expressing Ebola virus genes; (2) the vaccinia virus expressing Ebola glycoproteins; and (3) encapsulated, gamma-irradiated Ebola particles in lipid A liposomes.²¹⁰ There has also been Ebola vaccine experimentation with some success in nonhuman primate models, involving (*a*) using an adenovirus vector to deliver key glycoproteins, and (*b*) using DNA vaccine technology²¹¹ followed by boosting with an adenovirus vector.²¹² Recently, an attenuated recombinant vesicular stomatitis virus vector with either Ebola or Marburg glycoproteins demonstrated protection in nonhuman primate models.²¹³ Not only did the animals survive the challenge, but they also showed no evidence of Ebola or Marburg virus after challenge, nor evidence of fever or any adverse reaction to vaccination. However, none of the current vaccine candidates will be ready for licensure soon.

Antiviral Agents

Ribavirin. Antiviral medications prescribed to treat VHFs are important primarily after patients have developed symptoms, because there are insufficient data to support their use for postexposure prophylaxis. The medication with the most evidence of efficacy is ribavirin. Ribavirin is a nonimmunosuppressive

nucleoside-analogue with activity against a number of different viruses. The principal mechanism is inhibition of inosine-5'-phosphate (IMP) dehydrogenase, which converts IMP to xanthine monophosphate.²¹⁴ Suggestive data exist for using ribavirin to treat the arenaviruses and bunyaviruses.²⁰³ In particular, human studies suggest ribavirin is effective for treating hantavirus associated with hemorrhagic fever with renal syndrome (HFRS)²¹⁵ and Lassa fever.²¹⁶ It may also be effective for treating Crimean-Congo hemorrhagic fever (CCHF) and the New-World arenaviruses. Data supporting the use of ribavirin for HFRS are derived from a double-blind, placebo-controlled trial²¹⁵ demonstrating a reduction in mortality as well as decreased duration of viremia.²¹⁷ The largest observational study on CCHF, conducted by Mardani et al, noted that 97 of 139 patients (69.8%) with suspected CCHF receiving oral ribavirin survived, compared to an untreated historical control in which 26 of 48 patients (54%) survived.²¹⁸ In another recent study of CCHF by Ergonul et al, eight patients were treated with ribavirin, and all of these patients survived. However, in the same clinical context, 22 patients with CCHF were not treated

and had a mortality rate of 4.5%.²¹⁹ Ribavirin also has demonstrated in-vitro activity against CCHF.^{220,221}

Ribavirin appears to be effective for treating infection with both Old-World (Lassa fever) and New-World arenaviruses (South American hemorrhagic fever viruses).²²² In Lassa fever, human studies suggest that ribavirin decreases mortality, especially if administered within 7 days of infection (the case fatality rate was reduced from 55% to 5%).²¹⁶ Results from nonhuman primate studies also support this finding.^{223,224} Ribavirin may also have benefit in Argentine hemorrhagic fever,^{225,226} but a large, randomized clinical trial has not been conducted. Ribavirin appears to have benefit in a macaque model for Argentine hemorrhagic fever²²⁷ if therapy is initiated at the onset of symptoms. For animals that were treated at the onset of symptoms, initial improvement was observed in three of the four animals, with one animal dying early in the course of illness. However, the three infected monkeys that initially improved while on ribavirin subsequently developed a central nervous system infection that was fatal in two animals. This study and others suggest that ribavirin, which does not cross the blood-brain barrier,

TABLE 21-7

MEDICAL COUNTERMEASURES FOR VIRAL HEMORRHAGIC FEVERS

Virus	Vaccine	Passive Immunotherapy	Ribavirin as Potential Therapy
<i>Arenaviridae</i>			
Lassa	No	Mixed results	Yes
Guanarito (Venezuelan hemorrhagic fever)	No		Yes
Junin (Argentine hemorrhagic fever)	Yes*	Yes	Yes
Machupo (Bolivian hemorrhagic fever)	No		Yes
Sabia (Brazilian hemorrhagic fever)	No		Yes
<i>Bunyaviridae</i>			
Crimean-Congo hemorrhagic fever	No	Limited data	Yes
Hemorrhagic fever with renal syndrome	Yes [†]		Yes
Rift Valley fever	Yes [‡]		No
<i>Filoviridae</i>			
Ebola	No [§]	Mixed results	No
Marburg	No [§]	Mixed results	No
<i>Flaviviridae</i>			
Yellow fever	Yes		No
Kyasanur Forest disease	No		No
Omsk hemorrhagic fever	No		No

*Candid 1 live attenuated vaccine for Argentine hemorrhagic fever

[†]Hantavax (Korea Green Cross Corporation, Yongin-si, Korea) for hemorrhagic fever with renal syndrome from hantaviruses[‡]Investigational formalin-inactivated Rift Valley fever vaccine; live attenuated Rift Valley fever vaccine[§]Active development program with potential products being tested in nonhuman primate models

may be less useful for infections that have a propensity to infect the central nervous system.²²² An anecdotal report notes recovery from Bolivian hemorrhagic fever after treatment with ribavirin in two patients.²²⁸

Because of the probable efficacy of ribavirin for some of the VHFs, a consensus statement on the management of these viruses in a biological weapon scenario recommends that ribavirin be started empirically in all cases, until a better identification of the agent is achieved.²⁰³ In addition to the possible benefits in VHF cases, especially when therapy is commenced as close to the onset of symptoms as possible, ribavirin generally has manageable side effects (particularly anemia), making empiric therapy preferable. Ribavirin is not effective against filoviruses or flaviviruses that cause VHFs²²² and should be discontinued if one of these viruses is identified as the causative agent. Although ribavirin is considered teratogenic and is contraindicated in pregnancy, the consensus statement suggests that ribavirin should be used in a biological weapon scenario because the benefits of treatment would likely outweigh the fetal risk.²⁰³ The group recommends clinical observation of exposed patients, with careful observation for fever or other signs and symptoms of infection, rather than using ribavirin for postexposure prophylaxis.²⁰³

The dose of ribavirin for a contained casualty scenario is as follows: one loading dose of 30 mg/kg (maximum 2 g), followed by 16 mg/kg intravenous (maximum 1 g per dose) every 6 hours for 4 days, followed by 8 mg/kg intravenous (maximum 500 mg per dose) every 8 hours for 6 days.²⁰³ In a mass-casualty situation, oral ribavirin is recommended. No other antiviral medications have been licensed or advocated for widespread use for the treatment of VHFs in a current casualty situation.

Other drugs. Few other options exist for treating VHFs, other than supportive care. Using steroids to treat these viruses is not recommended.²⁰³ Pathogenesis studies with Ebola virus have implicated tissue-factor-induced disseminated intravascular coagulation as a critical component of the fatal outcomes.²²⁹ In an Ebola-infection model, treating rhesus macaques with a factor VIIa/tissue factor inhibitor (recombinant nematode anticoagulation protein c2 or rNAPc2) led to a survival advantage.²³⁰ This compound has not been tested in humans for treating Ebola infection, and tissue factor inhibitors have not been effective in the treatment of septic shock.²³¹ Other antiviral compounds have been studied for viruses such as CCHF, and in-vitro data suggest that the Mx family of proteins may have antiviral activity against ribonucleic acid viruses, but further study is needed.²³² IMP dehydrogenase inhibitors (similar to ribavirin) have been tested in both in-vitro and animal models against arenaviruses, but these products have not yet been tested in humans.²³³

Other compounds that have demonstrated in in-vitro activity against arenaviruses include 3'-fluoro-3'-deoxyadenosine,²³⁴ phenothiazines,²³⁵ and myristic acid compounds.²³⁶ Several antivirals have been tested in a bunyavirus (Punta Toro virus) murine model,²³⁷ suggesting possible compounds for further testing.

Stimulating the immune system is another potential therapeutic modality, but no human studies with this technique have been conducted for any of the VHF viruses. Interferon combinations may be useful, particularly with VHF infections in which the immune response is impaired. However, interferon compounds may be deleterious in some VHF infections, such as Argentine hemorrhagic fever, in which high interferon levels are associated with worse outcomes.²³⁸ Interferons have demonstrated a benefit in bunyavirus murine models,²³⁷ and a slight benefit in a nonhuman primate Ebola virus model (using interferon α -2b).²³⁹

Passive Immunotherapy

Studies on the benefits of passive immunotherapy for treating VHFs have yielded mixed results.²⁰³ Sera collected from donors after infection with Argentine hemorrhagic fever have been used in the treatment of this disease.²²⁵ However, as with passive immunotherapy for treating other diseases, concerns about the transmission of bloodborne pathogens such as hepatitis C²⁴⁰ may limit this treatment, or at least necessitate a rigorous screening process. In a cymologous monkey model of Lassa fever infection, treatment with sera from immune monkeys led to a survival advantage when the sera was used alone and combined with ribavirin.²²⁴ However, sera from convalescent patients used to treat Lassa fever did not reduce mortality in patients with a high risk of a fatal outcome.²¹⁶ Anecdotal evidence suggests that immunoglobulins and/or transfusions from convalescent patients may improve outcome in human Ebola virus infection.^{241,242} Passive treatment with immunoglobulins did not produce a mortality benefit in a macaque model for Ebola virus infection.²³⁹ Substantial supportive data are lacking for using immunoglobulin from survivors for treating CCHF, but a small case series has suggested 100% survival among treated patients.²⁴³ Serum from vaccinated horses has also been suggested as being beneficial for CCHF.²⁴⁴

In addition to questions about the safety of donated sera, the impracticality of obtaining large quantities of donated sera from previously infected individuals, with no such population available (particularly in the United States), limits the utility of this treatment. Future technology, such as a means of manufacturing large quantities of monoclonal antibodies, may allow for passive treatment with antibodies to counteract the effects of VHF.

Other Countermeasures

Good infection control practices, particularly the isolation of patients and barrier precautions, are a crucial countermeasure in the efforts to limit the impact of VHF used as biological weapons. The specific infection control needed for each virus is discussed in chapter 13, Viral Hemorrhagic Fevers. Management measures must also overcome the fear and panic associated with use of a VHF virus such as Ebola.²⁴⁵

Modern intensive care unit support will likely improve the outcome for patients infected with VHF

viruses, but access to this care may be limited in a mass casualty scenario. For HFRS, the intensive care management is both crucial and challenging; access to dialysis can save lives because the renal failure associated with this infection tends not to be permanent. Fluid management must be carefully followed in HFRS because capillary leak syndrome constitutes one of the primary mechanisms of pathogenesis, and fluid replacement leads to increased pulmonary edema.²⁴⁶ The effects of various interventions (including blood products such as fresh frozen plasma and fluids) have not been adequately delineated and merit further study.

TOXINS

Botulinum Toxin

Clostridium botulinum is an anaerobic gram-positive bacillus that produces a potent neurotoxin, botulinum toxin. Botulinum toxin blocks the release of neurotransmitters that cause muscle contraction, and may result in muscle weakness, flaccid paralysis, and subsequent respiratory impairment. There are seven immunologically distinct toxin serotypes (A through G) produced by discrete strains of the organism. Botulism is generally acquired from ingestion of food contaminated with botulinum toxin, but may also occur from toxin production by *C botulinum* if present in the intestine or wounds. Botulism is not acquired naturally by aerosolization, and this route of acquisition would suggest a possible bioterrorism event but may also occur from exposure to aerosolized toxin in a research laboratory.²⁴⁷ Neurologic symptoms after inhalational of botulinum toxin may begin within 24 to 72 hours of the exposure, but may vary with exposure dose.

Vaccination

There are currently no FDA-approved vaccines to prevent botulism. However, an investigational product, the pentavalent botulinum toxoid (PBT) against botulinum toxin serotypes A through E has been used since 1959 for persons at risk for botulism (ie, laboratory workers).^{248,249}

Pentavalent Botulinum Toxoid. PBT is available as an investigational product on protocol through the CDC. Derived from formalin-inactivated, partially purified toxin serotypes A, B, C, D, and E, PBT was developed by the Department of Defense and originally manufactured by Parke-Davis Company. 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 indi-

vidual toxin serotypes were then blended to produce the end product. The Michigan Department of Public Health has been responsible for formulation of recent PBT lots.

PBT has been found to be protective in animal models against intraperitoneal challenge with botulinum toxin serotypes A through E, and protective in nonhuman primates against aerosol challenge to toxin serotype A.²⁵⁰ From 1945 until 1959, at-risk laboratory workers in the US offensive biological warfare program at Fort Detrick were vaccinated with a bivalent botulinum toxoid (serotypes A and B).²⁵¹ There were 50 accidental exposures to botulinum toxins reported from 1945 to 1969 (24 percutaneous, 22 aerosol, and 4 by ingestion), but no cases of laboratory-acquired botulism occurred, possibly attributed in part to protection from the botulinum toxoids. The PBT was initially given as a primary series of three subcutaneous injections (0.5 mL at 0, 2, and 12 weeks) and a booster dose at 12 months. Subsequent booster doses were required yearly, but later required only for a decline in antitoxin titers (antitoxin not present on a 1:16 dilution of serum). Antitoxin titers from vaccination with PBT generally do not occur until 3 to 4 months after initiation of the vaccine (1 month after the third dose), so postexposure vaccination with the PBT is not recommended.

Recent data suggest a declining immunogenicity and potency associated with increasing age of PBT, which was manufactured 30 years ago.^{252,253} Antitoxin titers obtained 1 month after booster doses of PBT given between 1999 and 2000 to at-risk USAMRIID laboratory workers were "adequate" (a predetermined antitoxin titer that allowed for deferment of a booster dose) for toxin serotypes A, B, and E in 96%, 73%, and 45% of vaccinees, respectively.^{252,253} Adequate titers obtained between 6 and 12 months after a booster dose were noted in only 76%, 29%, and 12% of vaccinees for toxin serotypes A, B, and E, respectively.^{252,253} These data suggested declining PBT immunogenicity,

because earlier data (from 1986 to 1990) demonstrated adequate titers to toxin serotypes A and B persisting for 1 year after a booster dose in 96% and 44% of vaccinees, respectively.²⁵⁴

The Harris study, conducted from 1998 to 2000, demonstrated that approximately two thirds of vaccinees had a decrease in antitoxin titers by week 24 (6 months).^{253,255,256} Studies of the PBT in 1963 demonstrated a decline in antitoxin titers occurring between week 14 and 52 (with most individuals not having measurable antitoxin titers at week 52), suggesting the need for a 6-month dose even with early PBT lots.²⁵⁷

Recent potency studies and antitoxin titers in 2005 have demonstrated that PBT may still offer potential protection against toxin serotype A, and to serotype B with lot PBP003. Potency studies demonstrated PBT still protects animals against challenge to toxin serotype C even though the PBT no longer produces adequate neutralizing antibody levels to toxin serotype C for passing potency testing. The PBT no longer provides adequate protection of animals (requires \geq 50% animal survival postchallenge with lethal dose of toxin) or produces adequate neutralizing antibody levels against toxin serotypes D and E.²⁵³

Until recently, PBT was 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 booster doses thereafter only for declining antitoxin titers.²⁵⁷ The PBT dosing schedule was changed in 2004 because of the recent decline in immunogenicity and potency, and because of the results of the Harris study. The protocol for PBT lots produced in the 1970s now requires a primary series of four injections (0.5 mL at 0, 2, 12, and 24 weeks). A booster dose is still given at 12 months because antitoxin titers from the 24-week dose declined again by month 12 in the Harris study, and booster doses are now required annually. The CDC's current recommendation for at-risk persons who have received lots of PBT made in the 1970s is to consider personal protective measures as the sole source of protection against all the botulinum toxin serotypes.

Adverse events. PBT has been demonstrated to be safe, with adverse events being mainly local reactions at the injection site. Data from the CDC (passively reported) from over 20,000 vaccinations from 1970 to 2002 showed mild or no reaction associated with 91% of vaccinations, moderate local reactions (edema or induration between 30 and 120 mm) with 7% of vaccinations, and severe local reactions (reaction size greater than 120 mm, marked limitation of arm movement, or marked axillary node tenderness) with less than 1% of vaccinations. Systemic reactions occurred in approximately 5% of vaccinees, and were nondebilitating and reversible (mainly general malaise, chills or fever, itching or hives, and soreness or stiffness of the neck or back).²⁵⁸

New vaccine research. Vaccine candidates include formalin-inactivated toxoids (A through F) made in nearly the same way as formalin-inactivated PBT, with the goal of FDA approval.^{259,260} However, production of formalin-inactivated toxoids is expensive and relatively time consuming. The production requires partially purified culture supernatants to be treated exhaustively with formaldehyde, performed by a highly trained staff within a dedicated high-containment laboratory space.²⁶¹ Furthermore, the resulting PBT is relatively impure, containing only 10% neurotoxin (90% is irrelevant material). This impurity may be partly responsible for the occurrence of local reactions as well as the need for multiple injections to achieve and sustain protective titers. A bivalent AB botulinum toxin was developed based on the experience of the PBT that optimized several of the manufacturing issues of the PBT, including a reduction of formaldehyde levels in the final product to potentially reduce local reactinogenicity.²⁶² Preclinical studies in the guinea pig and mouse models demonstrated that a single dose of 1.0 mL was protective against intraperitoneal challenge with toxin serotypes A and B, and it was associated with neutralizing antibody titers in guinea pigs of 8 IU/mL to toxin serotype A (50 to 100 times higher than generally observed with the PBT) and 1.25 IU/mL to toxin serotype B (10 to 20 times higher than observed with the PBT).

The use of pure and concentrated antigen in recombinant vaccines could offer advantages of increased immunogenicity and decreased reactogenicity (local reactions at the injection site) over formalin-inactivated toxoids.²⁶³ Recombinant techniques use a fragment of the toxin that is immunogenic but is not capable of blocking cholinergic neurotransmitters. 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 of the toxin. Vaccine candidates using recombinant fragments of botulinum toxins against botulinum toxin serotypes A, B, C, E, and F were protective in mice.²⁶³⁻²⁷² A vaccine recombinant candidate for botulinum toxin serotype A was protective in mice challenged intraperitoneally, producing levels of immunity similar to that attained with PBT, but with increased safety and a decreased cost per dose.²⁶¹ Phase I trials on the bivalent recombinant vaccine (toxin serotypes A and B) have been completed, with promising preliminary serologic results at 12 months after two doses of vaccine (at 0 and 6 weeks), and phase II trials are being proposed.²⁵³ Recombinant vaccines given by aerosol are also being investigated.^{273,274}

A candidate vaccine using a VEE virus replicon vector that involves the insertion of a synthetic carboxy-terminal fragment gene of the heavy chain of toxin serotype A is also being evaluated.²⁷⁵ This vaccine

induced a strong antibody response in the mouse model and remained protective in mice against intraperitoneal challenge at 12 months.

Postexposure Prophylaxis

Any individuals suspected to have been exposed to botulinum toxin should be carefully monitored for evidence of botulism. Botulinum antitoxin should be administered if a person begins to develop symptoms of botulism. The bivalent botulinum antitoxin (serotypes A and B) is the only FDA-approved antitoxin preparation for adults currently available. The trivalent equine botulinum antitoxin (serotypes A, B, and E) is no longer available at the CDC because of declining antitoxin titers to toxin serotype E in this product. However, botulinum antitoxin for serotype E is available as an investigational product at the CDC (an equine antitoxin) and the California Department of Public Health (a human botulinum toxin immune globulin).

BabyBIG, a human botulism immune globulin derived from pooled plasma of adults immunized with PBT (A through E), was approved by the FDA in October 2003 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 Department of Health Services (510-231-7600).

Additionally, USAMRIID had developed two equine antitoxin preparations against all toxin serotypes that are available as investigational use drugs for treating botulism: (1) botulinum antitoxin, heptavalent, equine, types A, B, C, D, E, F, and G (HE-BAT) and (2) botulinum antitoxin, F(ab')₂ heptavalent, equine toxin neutralizing activity types A, B, C, D, E, F, and G (Hfab-BAT). These products are "despeciated" equine antitoxin preparations, made by cleaving the F_c fragments from the horse immunoglobulin G molecules, leaving only the F(ab')₂ fragments. However, 4% of horse antigens are still present in the preparation, so there is still a risk for hypersensitivity reactions. These investigational products are for use for treatment of botulism, and they would be considered for prophylactic use in asymptomatic persons only in special, high-risk circumstances.

Although passive antibody prophylaxis has been effective in protecting laboratory animals from toxin exposure,²⁷⁶ 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.

Staphylococcal Enterotoxin B

Staphylococcal enterotoxins are toxins produced by *Staphylococcus aureus*, referred to as superantigens. Ingestion of staphylococcal enterotoxin B (SEB) is a common cause of food poisoning. However, inhalation of SEB may cause fever with respiratory symptoms within 3 to 12 hours of exposure, which may progress to overt pulmonary edema, acute respiratory disease syndrome, septic shock, and death.²⁷⁷ The binding of toxin to the major histocompatibility complex stimulates the proliferation of large numbers of T cells, which results in production of cytokines (tumor necrosis factor, interferon-gamma, and interleukin-1) that are thought to mediate many of the toxic effects.

Vaccination

No vaccine against SEB is currently 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.

New vaccine research is ongoing. 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.²⁷⁸ Subsequently, intramuscular vaccination with recombinantly attenuated SEB using an Alhydrogel (Accurate Chemical & Scientific Corporation, Westbury, NY) adjuvant was found to be protective in rhesus monkeys challenged by aerosols of lethal doses of SEB.²⁷⁹ All monkeys developed antibody titers, and the release of inflammatory cytokines was not triggered.

A candidate SEB vaccine using a VEE virus replicon as a vector has also been studied.²⁸⁰ The gene encoding mutagenized SEB was cloned into the VEE replicon plasmid, and the product was then assembled into VEE replicon particles. The vaccine elicited a strong antibody response in animal models and was protective against lethal doses of SEB.

SEB toxoids (formalin-inactivated) incorporated into meningococcal proteosomes or microspheres have been found to be immunogenic and protective against aerosol SEB challenge in nonhuman primates. The proteosome-toxoid given by intratracheal route elicited serum IgG and IgA antibody titers, and a strong IgA

response in bronchial secretions.²⁸¹ 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 toxoid, which results in both a primary and an anamnestic secondary antitoxin response and thereby may require fewer doses.)²⁸² However, enteric symptoms such as vomiting still occurred in many vaccinees with both vaccine candidates.²⁸¹⁻²⁸³

Postexposure Prophylaxis

No postexposure prophylaxis for SEB is available. Although passive immunotherapy can reduce mortality in animal models if given within 4 to 8 hours after inhalation, there are no current clinical trials in humans.

Ricin

Ricin is a protein toxin derived from the beans of the castor plant. Ricin's mechanism of toxicity is by inhibition of protein synthesis, which ultimately results in cell death. Inhalation of ricin as a small-particle aerosol may produce pathological changes 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 in severe gastrointestinal symptoms (nausea, vomiting, cramps, and diarrhea) followed by vascular collapse and death.

Vaccination

No vaccine is currently available, but several vaccine candidates are being studied.²⁸⁴ Because passive prophylaxis with monoclonal antibodies in animals is protective against ricin challenge, the vaccine candidates are based on induction of a humoral response.^{285,286} However, even a single molecule of ricin toxin A-chain (RTA) within the cytoplasm of a cell will completely inhibit protein synthesis,²⁸⁷ so any ricin toxoid may have the potential toxicity for vascular leak even if it is 1,000-fold less toxic.²⁸⁸ Therefore, although ricin intoxication in animals can be prevented by vaccination

with a formalinized ricin toxin (toxoid) or a deglycosylated RTA,²⁸⁹ there is still a concern and potential risk of vascular leak with these vaccine candidates.

The most promising development for a vaccine has been to genetically engineer the 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 protect mice against intraperitoneal challenge with large doses of ricin.²⁸⁴ A pilot clinical trial in humans demonstrated a recombinant RTA vaccine (RiVax) given as three monthly intramuscular injections at doses of 10, 33, or 100 ug (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.²⁹⁰ Further human trials with this vaccine are not planned due to vaccine instability.

A ricin vaccine candidate (RTA 1-33/44-198) developed at USAMRIID demonstrated high relative stability to thermal denaturation, no detectable cytotoxicity, and immunogenicity in animal studies.²⁹¹ The vaccine (given as 3 intramuscular injections at 0, 4, and 8 weeks) was protective in mice against aerosol challenge with ricin at doses between 5 and 10 times the LD₅₀.²⁹¹ Additionally, no toxicity was observed in two animal models.²⁹¹

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.²⁹² Oral vaccination of mice with the ricin toxoid vaccine encapsulated in poly(lactide-co-glycolide) microspheres was also protective against lethal aerosol ricin challenge.²⁹³

Postexposure Prophylaxis

There is no postexposure prophylaxis for ricin intoxication. 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.^{285,286}

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 pro-

tect both the military and civilian populations. The military has recognized the benefit of vaccinating troops for protection against exposure from 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. Risk–benefit assessments must be considered in vaccine recommendations for the civilian and military populations, as well as the logistics of maintaining immunity with vaccine booster doses. 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 distribution of medical supplies and management of bioterrorism events within the continental United States, and it is the responsibility that military physicians be properly trained and prepared for managing bioterrorism events.

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Chapter 22

BIOSAFETY

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INTRODUCTION

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BIOSAFETY PROGRAM ELEMENTS REQUIRED FOR CONTAINMENT AND MAXIMUM CONTAINMENT LABORATORIES

- Measures Taken in Research to Protect Laboratory Workers
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THE BIOSAFETY PROFESSION

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, 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.

The goal of handling these hazardous agents safely can be accomplished through careful integration of accepted microbiological practices, and the 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 through adherence to good 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 protection of 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. (Historically, the designation “P” was used to indicate the level of physical containment, such as P-1 through P-4.) The current terminology is biosafety level or BSL.¹ The designation BSL is used in the *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*,¹ which focuses on protecting laboratory employees. BL is another designation for biosafety level, 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 protective suits (space suit or blue suit) are used solely in a maximum containment, or BSL-4, laboratory. 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 were 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, development of safer working practices, principles, and engineering controls was needed.^{3,4} 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, Wedum⁴ 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 positive-pressure protective suit facilities when working with potential aerosol-transmitted zoonotic microorganisms (eg, those

- causing tularemia and Q fever if a class III cabinet system was not available); and
- vaccinations 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 Barkley⁸ 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, Inc (Boston, Mass), 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 af-

fixed 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 have been documented during the past years. 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, 0.5–5.0 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 agents used in research, are placed into risk groups based on the danger they pose to human health. The risk group as-

signment helps guide the researcher in determining the containment condition (or BSL) appropriate for handling any particular agent.

Multiple schemes for assigning risk groups have been developed. The *NIH Guidelines*; the American

Biological Safety Association (Mundelein, Ill); 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 unlikely to cause human or animal disease. 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 the causative agent of anthrax, *Bacillus anthracis*, 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. Effective treatment and preventive measures are 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 transmit-

ted 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. The relationship of risk groups and BSLs, practices, and equipment is illustrated in Table 22-1.

How Agents Are Placed in Risk Groups

To assess the risk while working in a laboratory or animal environment with a specific microorganism, the following criteria must be considered: number of past laboratory infections, natural mortality rate, human infectious dose, efficacy of vaccination and treatment, extent to which infected animals transmit the disease, stability of the agent, and potential for exposure of the investigator.

- Number of past laboratory infections: The most frequent cause of laboratory-associated infections in humans is the *Brucella* species. Extra caution must be taken when working with this agent because of its low 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 widely¹⁵ (Table 22-2).
- Human infectious dose: Working with an

TABLE 22-1
RELATIONSHIP 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	Good microbiological techniques plus protective clothing; biohazard sign	Open bench plus BSC for potential aerosols
3	Containment: BSL-3	Special diagnostic services; research	As level 2 plus special clothing, controlled access, 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 22-2
CASE-FATALITY RATE BY DISEASE

Disease (Untreated)	Organism	[Case-Fatality Rate]
Plague, bubonic	<i>Yersinia pestis</i>	[50%–60%]
Cholera	<i>Vibrio cholerae</i>	[50% or more]
Tularemia, pulmonary	<i>Francisella tularensis</i>	[30%–60%]
Anthrax, cutaneous	<i>Bacillus anthracis</i>	[5%–20%]
Tularemia, typhoidal	<i>Francisella tularensis</i>	[5%–15%]
Brucellosis	<i>Brucella</i> species (<i>melitensis</i>)	[2% or less]
Q fever	<i>Coxiella burnetii</i>	[1%–2.4%]

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 22-3). Although the literature contains information about the potential infectious dose for humans as extrapolated from animal data (see Table 22-3), an attempt to provide quantitative human infectious doses is not possible.¹⁶

- Efficacy of vaccination and treatment (if either of these is 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;
- *cercopithecine herpesvirus 1* (B virus)—valacyclovirhydrochloride (VALTREX; GlaxoSmith-Kline, Research Triangle Park, NC); and
- *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 (IND) protocol (in the United States) only for empirical treatment of hemorrhagic fever virus patients while awaiting identification of the etiological agent.
- Extent to which infected animals transmit the disease: This discussion involves the zoonotic

TABLE 22-3
HUMAN INFECTIOUS DOSE BY ORGANISM

Organism	Infectious Dose	Route of Exposure
<i>Vibrio cholerae</i>	10 ⁸	Ingestion ¹
<i>Yersinia pestis</i>	100–20,000	Inhalation ²
<i>Bacillus anthracis</i>	~ 1,300	Inhalation ³
<i>Brucella</i> species (<i>melitensis</i>)	10–500	Inhalation ²
<i>Francisella tularensis</i>	10	Inhalation ⁴
<i>Coxiella burnetii</i>	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 burnetii* (Q-fever). *Appl Biosafety*. 2006;11:32–41.

diseases or diseases that can be transmitted from animals to humans. These diseases include the following:

- those transmitted directly from animals to humans (eg, rabies);
- diseases 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).

There are exposure risks in laboratories in which infectious disease research involving use of animals may differ from the exposure risks encountered in microbiology laboratories. Within the microbiology laboratory, potentially hazardous conditions arise from the activities of the humans or from use of equipment within the laboratory. In the animal facility, the animals themselves may create hazards for the laboratory workers through the following means:

- 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, with contamination of the animal holding room, cage, bedding, equipment, or other fomites.

In a controlled laboratory environment, laboratory workers and animal handlers can also be infected by diseased or infected animals via animal bites; by handling contaminated animal waste and bedding; and during animal manipulation, surgery, or necropsy. 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:** The stability of an agent (microorganism) to environmental conditions, and susceptibility or resistance to disinfectants,

is a result of its internal and external chemical compositions. For instance, spores of the genus *Bacillus* are resistant to adverse environmental conditions and disinfectants because of the presence of dipicolinic acid (DPA [pyridine-2,6-dicarboxylic acid]) in their spore coat. DPA plays a significant role in the survival of *Bacillus* spores exposed to wet heat and ultraviolet radiation.¹⁷ Many viruses and bacteria are sensitive to environmental conditions and disinfectants because of the high lipid content in their outermost layer.

- **Potential for exposure of the investigator:** The particular activity of an investigator, laboratory technician, or animal handler must be considered when estimating risk. If the worker is using a needle and syringe to inoculate animals, the potential for autoinjection is possible. An animal bite or scratch is another risk that must be considered.

Biosafety Levels

BSLs are guidelines that have evolved to protect laboratory workers. 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 consists of 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 the BMBL.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.¹

Primary hazards to personnel working with these agents are accidental percutaneous or mucous membrane exposures and ingestion of infectious materials. 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. 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 locking doors, sealed penetrations

or penetrations capable of being sealed, and directional airflow. (Airflow is 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 may not be available. 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.

It is important to understand how microorganisms are placed in risk groups and how that knowledge is used to develop procedures and physical infrastructure design to contain these agents. Next, the functions of various laboratories in response to a bioterrorist threat, the BSLs that these laboratories generally use, and the organization of these assets into a network known as the laboratory response network (LRN) will be explored.

LABORATORIES IN THE LABORATORY RESPONSE NETWORK

Clinical Laboratories

Clinical laboratories are located in community hospitals, diagnostic centers, public health research institutes, and at the state government level for referral. Clinical laboratories operate at a minimum using BSL-2 principles and practices in a facility designed to support their operations. As appropriate, all of these laboratories follow procedures for work with (a) human blood or blood products or with other potentially infectious material,¹⁸ (b) materials with a potential for generating an aerosol,¹⁹ (c) chemicals in the laboratory²⁰ and support areas,²¹ and (d) tuberculosis-causing agents.²² The procedures followed are based on degree of risk.

In response to the bioterrorism incidents of 2001, the National Laboratory Response Network for Bioterrorism was created. Coordination for this effort was assigned to the Centers for Disease Control and Prevention (CDC). The LRN consists of public and private laboratories functioning together to provide timely and accurate diagnostic testing using CDC-approved methods. These laboratories are in compliance with requirements of the National Electronic Disease Surveillance System (CDC/Public Health Information Network, Atlanta, Ga)²³ and the Health Insurance Portability and Accountability Act of 1996.²⁴ The LRN links local, state, and federal agencies in a three-tiered structure (sentinel laboratories, reference laboratories, and national laboratories) with a central role for the public health laboratory.

Sentinel Laboratories

Sentinel laboratories (formerly level A laboratories) include hospital and community-based clinical laboratories. They test patient specimens using highly sensitive methods to rule out or refer microorganism isolates to a reference laboratory. The laboratories are configured as BSL-2 laboratories and follow BSL-2 laboratory practices and safety equipment criteria. Work with infectious or potentially infectious material is conducted in a class II BSC. Sentinel laboratories provide a presumptive diagnosis. They do not have a testing role for environmental specimens in an overt event, nor do they conduct postattack recovery sample analysis. Their role is to rule out suspected bioterrorism agents and to recognize and report any bioterrorism suspicion, incident, or inquiry to a LRN reference laboratory.

Reference Laboratories

The reference laboratory (formerly included level B and level C laboratories) is the confirmatory (rule-in)

laboratory, such as a state public health laboratory, state agency laboratory, certain private sector laboratories, or a large local public health laboratory. These laboratories receive specimens from sentinel laboratories in the LRN and use more specific methods to confirm the preliminary identification of an organism. Reference laboratories are the primary response laboratories for an overt bioterrorism event, and they assist in a laboratory response and recovery to a covert event. The laboratories are configured as BSL-2 laboratories, but follow BSL-3 laboratory practices and equipment criteria. In addition, they conform to the Association of Public Health Laboratories (APHL) and CDC approval process. As reference laboratories, they offer state-of-the-art confirmatory testing. They provide bioterrorism information and training for the sentinel laboratories, laboratory support to first responders, and environmental sample testing in an overt event. State public health, federal, and academic laboratories—with a capacity for advanced diagnostic testing (molecular assays) and the capability of toxicity testing and evaluating new tests/reagents—are also included as reference laboratories.

National Laboratories

National laboratories (formerly level D laboratories) use the most sensitive and specific methods for characterizing microorganisms, and include the CDC and the US Army Medical Research Institute of Infectious Diseases (USAMRIID) at Fort Detrick. These laboratories have highly specialized facilities for isolating and identifying, confirming, validating, and manipulating rare and extremely lethal organisms (eg, Ebola virus, Lassa fever virus, and smallpox virus) in maximum containment (BSL-4) laboratories. The missions of national laboratories differ from those of other laboratories, because their primary role is not patient care, but research. These CDC research efforts provide tools to combat the infectious diseases that are of risk to the general population. USAMRIID's research efforts are focused on protecting military personnel on the battlefield by providing a means of prevention, detection, and intervention to infectious diseases that are known to be "weaponized" or have the potential to be used as weapons. Despite the differences in mission for these two national laboratories, the methods they use to protect the researcher and the community from infection are very similar. National laboratories may continue analysis of environmental samples during an overt attack or in support of recovery operations.

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 are applicable 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) BSL-4 facilities do not currently exist.

Documenting Safety Procedures

The *Biological Safety Program Manual*²⁵ is a laboratory-specific manual that should include specific safety standard/standing operating procedures (SOPs), guidelines, and documents for the containment laboratory. These safety SOPs identify the special hazards of the laboratory and the procedures to abate or mitigate the associated risk. The SOPs or documents specify the following:

- laboratory entry and exit in detail;
- proper use of laboratory-specific safety equipment (eg, BSCs, sterilizers, passboxes, 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-specific training.

A compilation of existing SOPs, specifying how a laboratory worker would access the SOPs (on-line, paper copy in a binder, or both) is suggested. To meet

the specific training 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 (MSDSs) for the chemicals used in the laboratory. MSDSs for chemicals can be obtained from vendors' Web sites or from the institutional chemical hygiene officer.

Assessing Individual Risk

For each person working in a BSL-3 and BSL-4 research laboratory, that individual's 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 the earlier section on risk assessment and risk management. 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 agent 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, microisolator cages; ventilated cage racks; and ventilated, negative-pressure, HEPA-filtered rigid cubicles or flexible isolators). Where the hazard cannot be eliminated by physical means, the hazard can be managed by administrative controls that provide specific training on procedures. Examples of such procedures include

disposal of used injection needles without recapping them or use of 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 the use of PPE to protect against contact, 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, protective suits, 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 NSF International Standard / American National Standard for Biosafety

Cabinetry - Class II (laminar flow) biosafety cabinetry Standard 49²⁶ on initial installation, at least annually thereafter, or after every major repair or relocation of the cabinet. 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), 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. When class II cabinets that recirculate air to the laboratory are used in BSL-4 facilities operated by US Department of Defense (DoD) organizations or contractors in support of biological research, development, test, and evaluation operations, they must be field certified every 6 months.²⁷

When working within a class II BSC, the equipment and materials are arranged in a clean-to-dirty layout, with clean materials (uncontaminated materials) in the center of the work space and contaminated materials at one end of the work space within the cabinet and contaminated waste materials at the other end of the work space.²⁸ Class III cabinets are totally enclosed, ventilated, gas-tight cabinets. They provide the highest level of product, personal, and environmental protection, and are most suitable for work in BSL-3 and BSL-4 laboratories. They also provide absolute protection against respiratory exposure to infectious or toxic aerosols. 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. 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, with wearing of 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) microisolator cages for rodents that have filter tops; (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.²⁹ Rigid, ventilated, negative-pressure, HEPA-filtered, mobile animal transport carts have been developed at USAMRIID to isolate animals during transfer between containment animal facilities.³⁰ 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.³¹ Disposable clothing should not be reused. Reusable clothing is decontaminated, usually by autoclaving, before being laundered to prevent an exposure hazard to laundry workers.³² 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.

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, Del], 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. Gloves should be changed frequently, followed by thorough hand washing. Disposable gloves should not be reworn. To ensure protection when working with highly hazardous materials, double gloving (wearing two pairs of gloves) should be practiced. If the outer glove is punctured or torn, the protective skin barrier should still be maintained by the inner glove if it, too, 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 overgloves (eg, Kevlar [EI Du Pont de Nemours and Company, Wilmington, Del]; armored, stainless-steel mesh; or leather gloves) for additional protection.³³ 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.

When entering rooms housing infected animals, use of 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). 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. 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.¹⁹ Users of tight-fitting respirators must be fit tested annually 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.

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 powered air-purifying respirator (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 greater than 19.5% 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. For example, USAMRIID has established an empirically based policy to replace particulate filters after 80 hours of use. 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 overboots. 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, be enrolled in an employer-provided OSHA-compliant respiratory protection program,¹⁹ 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.

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, jumpsuits, 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 suit laboratories and BSL-4 animal facilities, personnel must remove all personal clothing, including undergarments, socks, shoes, and jewelry. Complete laboratory clothing—including undergarments, pants, shirts, jumpsuits, socks, and gloves—is provided for, and used by, laboratory workers. Some institutes opt to omit wearing undergarments in containment. Workers don a fully encapsulating positive-pressure protective suit supported by an umbilical-supplied air system. The suit can be fitted with integral protective overboots or with legs terminating in soft booties. If a suit of the latter design is used, the worker dons protective overboots inside the BSL-4 suit facility, after passing through an airlock equipped with a decontaminating chemical suit shower. When not in use, protective overboots are stored inside the BSL-4 facility. As of this writing, positive-pressure encapsulating suits 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 positive-pressure encapsulating suits.^{19,27}

Medical Surveillance

Medical surveillance comprises baseline and periodic (usually annual) studies, including the following:

- complete medical history,
- urinalysis,
- hematology,
- serum chemistry panel,
- serum protective antibody titers for specific disease agents,
- physical examinations, 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. This program 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 objectives³⁴:

- protection of workers against health and safety hazards in the work environment;
- proper placement of workers according to their physical, mental, and emotional abilities;
- maintenance of a pleasant, healthy work environment;
- establishment of preplacement health examinations;
- establishment of regular, periodic health examinations (medical surveillance);
- diagnosis and treatment of occupational injuries, exposures, and diseases;
- consultation with the worker's personal physician, with the worker's consent, of other related health problems;
- health education and counseling for workers;
- safety education for workers;
- identification of hazardous situations or finding the means to prevent or mitigate hazardous situations; and
- establishment of surveys and studies of the industrial environment for protection of 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 an encapsulating, positive-pressure protective suit. 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 or greater, workers must be enrolled in an employer-provided hearing protection program to comply with OSHA regulations.³⁵ The program requires employees to undergo initial baseline and annual surveillance audiometry, fitting, and training to use hearing protectors (ear plugs or muffs).

It is required that personnel receive initial familiarization training to wear the suit, as well as extensive, documented, tailored training provided by an assigned

mentor before a laboratory worker is considered proficient to work independently in BSL-4 containment. After demonstrating proficiency, the laboratory worker 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 air line 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.³⁶ 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. Generally, the visor fogs up 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 suit 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.

Vaccinations

The decision to vaccinate is based on a benefit-to-risk analysis or a risk-reduction analysis.³⁷ To justify use of a vaccine, the benefit from vaccination must outweigh any potential untoward effects of the vaccine. Benefits of vaccination include induction of specific humoral (antibody-based) and cellular immunity to a given infectious agent or toxin. Risks of vaccinations consist of local or systemic reactions.

Even the safest vaccine product has a likelihood of producing unwanted or unexpected side effects or an adverse event in a small percentage of the population receiving the vaccine.³⁷ For at-risk personnel—including laboratory workers—use of appropriate prophylactic vaccines can provide an additional level of protection.¹ Each institute should have a written policy that defines at-risk personnel, specifies the risks and benefits of specific vaccinations, and identifies appropriate prophylactic vaccine products. The requirements and recommendations should address the infectious agents known to be present or likely to be encountered in a given institute.

For all clearly identified at-risk personnel, licensed vaccines for which the benefits clearly exceed the risks should be offered.¹ Examples of licensed vaccines for

identified at-risk personnel at USAMRIID include those for protection against hepatitis B, yellow fever, Japanese encephalitis, rabies, anthrax, smallpox, and other orthopox virus infections.

Recommendations must be carefully considered for the following situations¹:

- giving less efficacious vaccines (eg, those with diminished immunogenicity or loss of potency);
- giving vaccines associated with high rates of local or systemic reactions (vaccines with safety concerns, such as excessive reactogenicity);
- giving vaccines that induce increasingly severe reactions with repeated use (vaccines that induce hypersensitivity reactions); and
- giving unlicensed vaccines under IND protocols.

IND vaccines used under a US Food and Drug Administration exemption for research and vaccination of laboratory personnel include the following³⁸:

- two Venezuelan equine encephalitis vaccines,
- Eastern equine encephalitis vaccine,
- Western equine encephalitis vaccine,
- pentavalent botulinum toxoid,
- Rift Valley fever inactivated vaccine,
- Q-fever vaccine, and
- tularemia vaccine.

Possible contraindications for subject participation in vaccination programs or for work within biocontainment laboratories³⁹ include the following medical issues:

- chronic, serious, or uncontrolled medical problems;
- acute or temporary medical conditions;
- autoimmune disorders;
- impaired immunity;
- conditions that may obscure recognition of adverse events from investigational vaccines;
- conditions that could lead to unpredictable behavior or collapse, leading to increased risk of an individual or coworker to exposure or medical emergency within a laboratory;
- untoward reactions to multiple vaccinations; and
- vaccine-specific contraindications.

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 of 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 include entry vestibules or personnel airlocks that feature two self-closing and lockable doors, clothes change rooms and shower facilities, and a hand-washing sink in each individual laboratory room. The sink is located near the room exit door and has hands-free operation (using foot pedals, or knee/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 any 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. 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, “tell-tail”) should be available at the laboratory entry.

In animal BSL (ABSL)-3 facilities, room fittings and ventilation should be in accordance with the *Guide for Care and Use of Laboratory Animals (The Guide)*⁴⁰ and the BMBL.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 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, nonrecirculating ventilation system with supply and exhaust components balanced to ensure directional airflow from areas of lower potential hazard to areas

of higher potential hazard. Also required is 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), and an 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. 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). Required at the containment barrier is an autoclave with two interlocked doors with the outer door sealed to the outer wall (a so-called bioseal). The autoclave is automatically controlled so that the outer door cannot be opened until a sterilization cycle has been completed. Also provided is a dunk tank, fumigation chamber, or a ventilated equipment airlock for passage of materials into the containment area and safe decontamination and removal of materials that cannot be steam sterilized 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*.⁴⁰ 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 (see <http://www.epa.gov/pesticides/factsheets/antimic>). 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: (1) hospital and (2) 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.

An example of a liquid sterilant-disinfectant is Alcide EXSPORE (Alcide Corporation, Redmond, Wash) 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.⁴¹ 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—which 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.⁴²

An example of a general (household) disinfectant is Clorox Regular Bleach (The Clorox Company, Oakland, Calif; 6.00% sodium hypochlorite; EPA Registration No. 5813-50). When mixed at the rate of ¼ cup per gallon of water, it is labeled to kill listed microorganisms (specified viruses, fungi, and nonspore-forming bacteria).⁴³ 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>).

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. Steam sterilization of laboratory sewage may be either a continuous flow or a batch process. 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 potentially 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).⁴⁴ 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).⁴⁵

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. 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.

Additional standard practices common to BSL-1 through BSL-3 facilities are as follows:

- 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.⁴⁶ 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 at all times.
- Do not allow people who are at a heightened risk of becoming infected (eg, immunocompromised individuals) access to the laboratory or animal room when work with infectious agents is in progress. 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 are to receive appropriate vaccinations and skin tests, if available for the agents being handled or agents potentially present in the room.
- Store baseline and periodic serum samples collected from at-risk personnel. At intervals, collect and analyze serum samples from at-risk personnel working in ABSL-4 containment and communicate the results to those at-risk personnel.
- 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/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. Use syringes that resheath the needle and systems without needles. Dispose of used sharps in conveniently located puncture-resistant containers.
- Place all potentially infectious materials in covered, leakproof 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 a unbreakable, sealed secondary container. Pass this enclosed material through a chemical disinfectant dunk tank, fumigation chamber, or airlock having 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. 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, plants, and clothing 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.
- 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.⁴⁶
- 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 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.
- Autoclave or decontaminate all materials other than materials to be retained in a viable state before removing them from the BSL-4 facility.
- In BSL-4 containment, establish a system to report laboratory accidents and exposures, employee absenteeism, and medical surveillance of a potential laboratory-acquired illness.
- 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.

In ABSL-4 containment, personnel assigned to work with infected animals should work in pairs. Appropriate procedures should be used to reduce possible exposure to infectious agents.

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. While 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 22-1), 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 (are) 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 perception of their 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/guidelines. The ultimate objective of these goals is to keep everyone healthy while supporting productive research. Personnel training is paramount. Both initial and refresher training of personnel 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



Fig. 22-1. Institute personnel are depicted as the spokes of a wheel that work together to accomplish a common mission.

in the dialogue, which, for the most part, will result in better adherence to compliance of institute and regulatory policies.

The philosophy of a biosafety program is based on an early estimation of risk, followed by application of appropriate containment and protective measures. 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. Concluding remarks for each training session should reiterate the description of some obvious hazards and how safety personnel try to minimize the risk of these hazards.

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. Safety personnel must actively engage with and seek the help of all administrative and laboratory personnel in hazard identification. 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

policies through an integrated program of safety engineering, vaccination, health surveillance, and medical management of illness.

Risk encompasses an awareness of the risk, an assessment (or evaluation) of the risk, and management of the risk. Communication is a fundamental part of risk assessment and training.

The US Government developed a five-step risk management process (Figure 22-2).⁴⁷ The five sequential steps of the risk management process include the following:

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, the controls implemented are reviewed to determine whether they were adequate, or if additional controls must be added.

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. The terms survey (comprehensive view) and inspection (a critical appraisal,

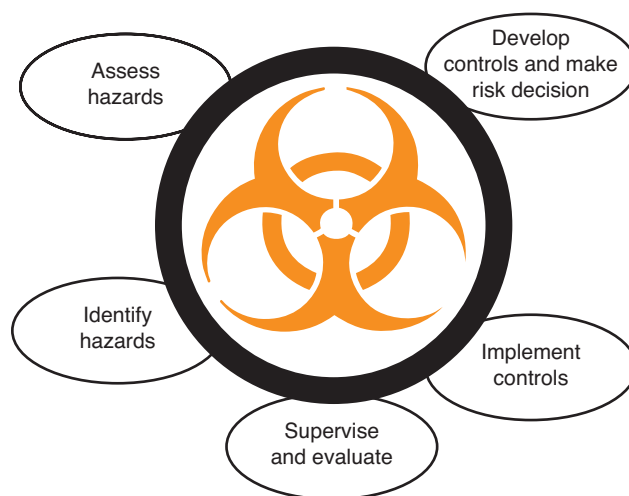


Fig. 22-2. Five steps of the risk management process. Adapted from: US Army Safety Center, Fort Detrick, Maryland.

an official examination, or checking or testing against established standards) are often used interchangeably with the term audit. 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.⁴⁸ 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. 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.⁴⁸

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 covered⁴⁹:

- autoclave repair and operational records,
- 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 laboratory signage,
- current certification of BSCs, and
- use of in-line HEPA filters on laboratory vacuum outlets.

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, un-scheduled audit of a laboratory include the following⁵⁰:

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 urgent time for evaluation of biosafety SOPs may be before a major outside organization or agency conducts a site visit.⁵⁰ 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 CDC and USDA-Animal and Plant Health Inspection Service (APHIS) Select Agent Program Laboratory Inspection Programs. For subordinate laboratories of the US Army Medical Research and Materiel Command, safety office personnel conduct periodic safety site assistance visits.²⁷ For DoD research, development, test, and evaluation (RDTE) laboratories, the director of Army safety conducts biological defense safety evaluation site visits.²⁷

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.²⁷ 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.²⁷ Safety officials maintain safety inspection records for 3 years,

and they review records annually to note trends that require corrective actions.²⁷ 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 and Radiation Protection conduct quarterly comprehensive inspections of BSL-1, BSL-2,

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.⁵¹

SELECT AGENT PROGRAM

Legislation for the CDC Select Agent Program was initially enacted in 1996 to document transfers between microbial culture and toxin repositories and laboratory facilities of certain pathogens infectious to humans and biological toxins injurious to humans, termed select agents and toxins.^{52,53} The objective of the legislation was to prevent transfer of these restricted biological materials to unauthorized individuals and facilities having the intent to use them for potentially nefarious purposes. The legislation established the original list of agents and required such facilities to be registered with the Department of Health and Human Services before transfers of restricted biological materials could be made to other registered facilities within the United States. As established, this Select Agent Rule, codified in Title 42 CFR Part 72.6,⁵³ also required an initial, periodic inspection of each registered facility to ensure that safety criteria were met. After the terrorist events of September 2001 and the ensuing anthrax-by-mail incidents shortly thereafter, the Patriot Act, enacted in 2001,^{54,55} and the Public Health Security and Bioterrorism Preparedness and Response Act of 2002 (Bioterrorism Act)^{56,57} extended the jurisdiction of control of the Select Agent Rule from facilities and individuals that only transfer select agents and toxins to all facilities and individuals that store and use select agents and toxins. The purpose of the new legislation was to protect against misuse of select agents and toxins whether inadvertent or the result of terrorist acts against the US homeland or other criminal acts. The codified regulations developed to implement the legislation^{46,58} ensured appropriate availability of biological agents and toxins for legitimate biomedical, agricultural, or veterinary research; education; and other purposes (while excluding their availability for illegitimate applications). The original list of biological materials was greatly expanded.

All laboratories (“entities”) having listed specified select agents and toxins, USDA select agents, overlap agents (agents appearing on both of the preceding lists), or listed plant pathogens must, by federal law, register each of their biological material holdings with the Department of Health and Human

Services, CDC,⁴⁶ or APHIS (Agricultural Bioterrorism Protection Act of 2002).^{58,59} Within each registered entity, a designated “responsible official” (entity owner, director, commander, or other designee within management), alternate responsible official(s) (to act in the absence of the responsible official), principal investigator(s), and staff member(s) having direct, unescorted access to the restricted biological materials must be named individually in the entity’s registration application to the CDC or to APHIS. All named individuals must undergo a successful security risk assessment conducted by the Federal Bureau of Investigation before these individuals are approved by the CDC or APHIS for unescorted access to an entity’s biological materials. As part of the entity registration process, inspectors from the CDC or APHIS will visit the entity with or without prior notice to inspect biological safety and physical security features of the laboratory facility, records of training, and health surveillance for personnel who have access to the restricted biological material, and also inventory records of all registered biological materials maintained by a given entity. In the inventory record, each registered biological material is listed by name, along with its location of storage and use and the name of the principal investigator. Inventory records must document each approved transfer and destruction of the biological materials, and must account for possession, propagation, and consumption in the course of bona fide mission work. Written regulations, protocols, and operating procedures (the so-called *Biological Safety Manual*) pertaining to work with the regulated biological material in the registered laboratory areas are examined. Also inspected are electronic security measures and the emergency response plan, including steps taken to report and recover lost, stolen, or diverted biological material. An entity registration has to be renewed every 3 years, and an individual’s security risk assessment has to be repeated every 5 years. Felony convictions for violations of the Select Agent Rule legislation can result in substantial sentences, including heavy fines and lengthy prison terms. The Select Agent Final Rule was published in 2005.^{45,58}

Biological Defense Research Program Laboratories

All laboratories involved in DoD RDTE operations must comply with the Department of the Army Biological Defense Safety Program.²⁷ 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. This regulation predates the Title 42 CFR Part 73,⁴⁶ but shares many features with the select agent program regulation and CDC/NIH guidelines.¹ The DoD Biological Surety (Biosurety) Program is a new program implemented in DoD biological defense RDTE laboratories that use DoD-provided biological agents.⁶⁰ This biosurety program is patterned after existing nuclear and chemical surety programs. The purpose of the biosurety program 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).^{60,61} One provision implements a two-person rule when working with biological select agents and toxins (BSATs), accomplished by having two individuals physically present in the laboratory room during work with these materials. This requirement can also be met by using surveillance cameras and random observations by roving observers. Physical security measures include inspection of all personal belongings on entry into the laboratory building and random exit inspections, as well as physical security upgrades to harden the laboratory building. Biological safety refers to the provisions of the Department of the Army Biological Defense Safety Program. Agent accountability consists of ensuring that documentation exists for storage and access to BSATs. A biological personal reliability program (BPRP), required by the biosurety program, exceeds the scope of the CDC Select Agent Program.⁴⁶ In addition to the security risk assessment required by the CDC Select Agent Program, the BPRP requires a background security investigation conducted at the level of that needed for a secret clearance for all individuals who work with or potentially have access to BSATs. The BPRP also requires initial and periodic urinalyses for illegal drug use and continuous medical and suitability screenings for as long as an individual remains enrolled in the BPRP.

Laboratory Animal Care and Use Program

Federal animal welfare regulations^{62,63} (AWRs) from USDA and APHIS, state and local laws, and the Public Health Service Policy on Humane Care and

Use of Animals⁶⁴ 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*⁴⁰ (the *Guide*). 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 to ensure that they are consistent with the AWRs, Public Health Service policy (for those institutions that receive NIH funding), and recommendations specified in the *Guide*. This 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, AAALAC International 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 International, 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 International is mandatory for DoD organizations and facilities maintaining animals for use in DoD programs.⁶⁵

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, 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 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.⁶⁶ An example of an academic fellowship program is the biosafety fellowship program at Washington University School of Medicine in St. Louis, Missouri.

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 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.⁶⁷ 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 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, and local regulations and guidelines.

To achieve the goals of the biosafety program, information pertaining to the program must be conveyed to the work force, along with how it benefits the work

force. 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 work force 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 following⁶⁸:

- 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; and
- using PPE appropriately (always).

Management must consider safety a top priority and work closely on a daily basis with safety professionals, who need management's 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

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Chapter 23

BIOSURETY

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INTRODUCTION

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INTRODUCTION

The influence of infectious disease on the course of history has been continuous. Endemic diseases such as malaria and human immunodeficiency virus have contributed to the endemic poverty of many Third World countries. Although humans have coexisted with infectious diseases for centuries, their potential for use as weapons against humans has become a matter of particular concern. Use of infectious diseases against enemies is not a new idea. Throughout history there have been well-documented and deliberate attempts to use noxious agents to influence battles, assassinate individuals, and terrorize the masses. South American aboriginal hunters often use arrow tips dipped in curare and amphibian-derived toxins. Additionally, there are reports from antiquity that crude wastes and animal carcasses were catapulted over castle walls and dropped into wells and other bodies of water to contaminate water sources of opposing forces and civilian populations. These practices precede written records but demonstrate the human race's long involvement in the use of biological weapons. One of the earliest well-documented cases of using infectious agents in warfare dates back to the 14th century siege of Kaffa (now Feodosia, Ukraine). During the attack, the Tartan forces experienced a plague outbreak. Turning their misfortune into advantage, they began to hurl the cadavers of the deceased into Kaffa using a catapult. Defending forces retreated in fear of contracting the plague. The abandoned city was easily taken by the Tartan forces, and the hasty retreat from Kaffa resulted in the spread of the plague epidemic to Constantinople, Genoa, Venice, and other Mediterranean port cities where the retreating forces found safe harbor.¹⁻³

Tactics such as these, and the understanding that disease, or even fear of disease, can be as detrimental to fighting forces as bullets, led military leaders to seek ways in which they could prevent disease among their soldiers as well as use it against their enemies. Although the first vaccine for smallpox was not used until 1796, variolation was practiced long before that time and provided lifelong immunity. Variolation was the procedure of deliberately inoculating people using scabs from smallpox infections either blown into the nose or rubbed into a puncture on the skin. General George Washington ordered the variolation of all soldiers in 1777. Because they were able to protect their own forces, commanders were free to use infectious disease in more deliberate ways. The British military reportedly used smallpox as a weapon against the Delaware Indians when General Jeffery Amherst ordered that blankets and handkerchiefs from smallpox-infected patients at Fort Pitt's infirmary be presented

to them during a peace meeting.^{1,2,4,5}

During the 19th century there were many advances in the understanding of bacterial agents. For the first time bacteria were isolated from diseased individuals and animals and grown in artificial culture outside the body using various growth media. Armed with these new methods of growing large volumes of bacteria, German scientists and officers began a large biological campaign against the Allied Forces during World War I. Instead of targeting the soldiers in this campaign, they targeted the livestock that were destined for shipment to the Allied Forces with the agents causing anthrax and glanders. Large numbers of horses and mules were reported to have died from these infections.^{1,2,6,7} These biological campaigns are considered to have had a negligible effect on the outcome of the war. The Germans were far more successful in their campaigns with chemical agents.

The devastating effects of German chemical warfare efforts led to the drafting of the Protocol for the Prohibition of the Use in War of Asphyxiating, Poisonous or Other Gases and of Bacteriological Methods of Warfare, signed at Geneva, Switzerland, on June 17, 1925.^{8,9} This treaty prohibited the use of both biological and chemical agents in warfare but did not provide for any inspections to verify compliance. Nor did the treaty prohibit the use of biological or chemical agents in research, production of agents, or possession of biological weapons. Many countries agreed to the measure in 1925 with the stipulation that they had the right to retaliate against biological or chemical weapon attacks with their own arsenals. Many countries proceeded to work with both biological and chemical weapons, and 50 years passed before any agreement on biological and toxin weapons was ratified by the US Senate. The Japanese aggressively advanced biowarfare in World War II by using Chinese prisoners to study the effects of anthrax, cholera, typhoid, and plague. More than 10,000 people were killed from the use of these agents on both military prisoners and civilian populations.^{1,2,10} Despite their best efforts at the time, the Japanese never developed an effective means of infecting large numbers of persons using biological munitions.

By the end of World War II, the Americans and Soviets were investing heavily in the weaponization of biological agents. Advances in science and technology allowed researchers to develop efficient ways to disperse infectious agents, often using routes quite different from the way people normally contracted the disease. Infectious agents were placed in missiles, bombs, and aerosol delivery systems capable of targeting large numbers of people. The ability to create

aerosol clouds of infectious disease agents and infect large numbers of people simultaneously changed the perceived risk associated with biological agents. Scientists estimated that casualties caused by the release of agents from aircraft ranged from 400 to 95,000 dead and 35,000 to 125,000 incapacitated depending on the agents used.^{2,11} Agents that had been encountered only in manageable, naturally occurring outbreaks acquired the potential to kill or incapacitate large numbers of people.

The lethal and unpredictable nature of biological weapons and their ability to affect noncombatants galvanized the global community against their use in warfare, and led to over 100 nations, including the United States, Iraq, and the former Soviet Union, signing the 1972 Biological Weapons Convention.^{9,12} This treaty prohibited the use of biological agents as weapons but stopped short of ending defensive research. The ability of some countries to continue aggressive weapons development programs despite having signed the convention demonstrated its ineffectiveness as a means of controlling the proliferation of biological and chemical weapons. The United States eventually withdrew as a signatory of this convention in 2001; however, it still holds to the basic tenets of the agreement.

President Richard M Nixon ordered the dismantling of the US offensive biological weapons program and diverted its funding to other vital efforts such as cancer research in 1969. Although the United States and Great Britain were busy destroying their weapon stockpiles, other countries and extremist organizations continued to develop and use both biological and chemical weapons. In the 1970s the Soviet Union and its allies were suspected of having used “yellow rain” (trichothecene mycotoxins) during campaigns in Laos, Cambodia, and Afghanistan.¹ An accidental release of *Bacillus anthracis* spores (the causative agent of anthrax) from a Soviet weapons facility in Sverdlovsk killed at least 66 people in 1979.¹³⁻¹⁵ After the Persian Gulf War and United Nations Special Commission inspections, Iraq disclosed that it had bombs, Scud missiles, 122-mm rockets, and artillery shells armed with botulinum toxin, *B anthracis* spores, and aflatoxin. According to a 2002 report from the Center for Nonproliferation Studies, six countries (Iran, Iraq, Libya, North Korea, Russia, and Syria) were known to possess biological or toxin weapons based on clear evidence of a weaponization program. An additional 11 nations (Algeria, China, Cuba, Egypt, Ethiopia, Israel, Myanmar, Pakistan, Sudan, Taiwan, and Vietnam) were suspected of having biological weapons programs with varying certainty. This list includes nations that also had former weapons programs.¹⁶ Because of the lack of verification in any of

the international agreements, it is difficult to determine whether the massive quantities of agents produced by those nations have been destroyed. Although the Biological Weapons Convention attempted to restrain nations in the biological weapons race, other events make it clear that the greater threat may now come from extremist organizations that exploit political instability worldwide to gain access to the agents and technologies that will further their agendas.

Extremist organizations have used biological agents to further their agendas since the 1980s. Food and water contamination may be a highly effective means to deliver a chemical or biological attack. Over 750 people were infected with *Salmonella typhimurium* through contamination of restaurant salad bars in Oregon by followers of the Bhagwan Shree Rajneesh in 1984.^{1,2,17} A Japanese sect of the Aum Shinrikyo cult attempted an aerosolized release of the anthrax agent from Tokyo building tops in 1994.^{1,2,18} This cult also unsuccessfully attempted to obtain Ebola virus during an outbreak in Africa during the 1990s, and it released sarin nerve gas into a subway system in Tokyo. Several national and international groups have been found in possession of ricin toxin with the intent to disperse the toxin in an attack.^{1,2} The anthrax mailings sent in October 2001 in the United States demonstrated that individuals were able to use biological agents as bioterrorism experts had warned for more than two decades. Although the anthrax attacks were not successful in causing large numbers of casualties and fatalities, they did have a significant economic and emotional impact. The Centers for Disease Control and Prevention (CDC) reported the effects of this one attack included 5 fatalities, 17 illnesses, a cost of \$23 million to decontaminate one Senate office building, \$2 billion in lost revenue to the US Postal Service, and as much as \$3 billion for the decontamination of the US Postal Service buildings and procurement of mail sanitizing equipment.¹⁹

As the potential use of these agents by extremist organizations and individuals came into the spotlight, congressional interest in regulating the research community increased. It was evident that a fundamental change in the US policy toward the regulation of these agents was required. The need for change was made apparent by the case of Larry Wayne Harris, microbiologist and suspected white supremacist, who was arrested in 1995 after receiving freeze-dried cultures of *Yersinia pestis* (the agent that causes plague) from the American Type Culture Collection. Because it was not a crime to possess these materials, he was only able to be charged for mail fraud and sentenced to 18 months of probation and 200 hours of community service in spite of the fact that there was a clear intent to use these materials in a malicious manner. At the time that his

crime was committed, it was not a federal offense or even illegal to be in possession of these agents.²⁰ In contrast, once the laws were changed, a professor in Texas who was conducting valid research without malicious intent was convicted and sentenced to 2 years in prison for improper handling of plague samples. The prosecutor in the case was seeking 10 years in prison and millions in fines; however, the sentence was reduced because of the great contributions that Thomas Butler had made to the scientific community. There was no indication that he planned on using these specimens for bioterrorism.^{21,22} Since that conviction, there has been concern in the scientific community regarding the risks of engaging in research that could put one in jail for relatively minor infractions of the law.

The US government and other nations have undertaken a variety of approaches to combat the extremist threat. Export controls on key precursor materials and equipment have been implemented since 2001. New

technical sensors to detect and identify specific agents or categories of agents have been developed and deployed. These systems have been used during events where large populations have assembled such as the Olympic games and the Super Bowl. In direct response to the anthrax mailings of 2001, the US Postal Service has implemented a continuous surveillance of major distribution centers to protect both their workers and the general public from another attack. New systems to monitor public health, such as syndromic surveillance systems, have been developed. Syndromic surveillance assists in highlighting areas in which an epidemic or outbreak might occur so that a containment and treatment strategy can be developed. Finally, to prepare for situations in which detection and surveillance efforts fail to warn of an attack, agencies in the federal government are focusing efforts to develop, improve, and stockpile medical countermeasures to the recognized biowarfare threat agents.²³

REGULATORY AGENCIES

After the Oklahoma City bombing, Congress passed the Anti-Terrorism Act of 1996. This act provides law enforcement activities with a broad range of new tools to be used in investigating and prosecuting potential acts of terrorism in the United States. With this act, Congress declared that the responsibility for developing regulations to control access to and possession of biowarfare threat agents would be the US Department of Health and Human Services (DHHS) and the US Department of Agriculture (USDA).

The first regulatory framework for working with and transferring select agents and toxins was published by the CDC in 1997. In these regulations the CDC had four goals:

1. identify the agents that are potentially hazardous to the public health;
2. create procedures for monitoring the acquisition and transfer of the restricted agents;
3. establish safeguards for the transportation of these infectious materials; and
4. create a system for alerting the proper authorities when an improper attempt is made to acquire a restricted agent.

In June 2002, the CDC convened an interagency working group with diverse representation, including Department of Defense (DoD) experts, to determine which infectious diseases and toxins should be listed as select agents requiring regulation.

On December 13, 2002, DHHS and the USDA each

published interim regulations in the *Federal Register* that addressed the possession, use, and transfer of select biological agents and toxins (select agents). The final rule, which was published on March 18, 2005, is updated periodically to include emerging threats. The DHHS regulations are published in Title 42 Code of Federal Regulations (CFR) Part 73,¹⁹ and the USDA regulations are published in Title 7 CFR Part 331²⁴ and Title 9 CFR Part 121.²⁵ These rules apply to all academic institutions and biomedical centers; commercial manufacturing facilities; federal, state, and local laboratories; and research facilities. Regulated agents and toxins appear in Chapter 18, Laboratory Identification of Biological Threats, Exhibit 18-1.

The original list published in December 2002 remains largely unchanged in the regulation, which was published on March 18, 2005. The list is not limited to the infectious agent or toxin itself but also regulates the agents' genetic elements, recombinant nucleic acids, and recombinant organisms. If the DNA or RNA of an agent on the listing can be used to recreate the virus from which it was derived, then the genetic material is also subject to the regulation. Any organism that has been genetically altered must also be regulated. Finally, recombinant nucleic acids that encode for functional forms of toxins that can be expressed in vivo or in vitro are subject to regulation to safeguard this material.

Some notable exceptions to the regulation allow for the unencumbered handling of diagnostic specimens by clinical laboratories. Title 42 CFR 73.5 states:

“Clinical or diagnostic laboratories and other entities that possess, use or transfer a DHHS select agent or toxin that is contained in a specimen presented for diagnosis or verification will be exempt from the requirements of this part for such agent or toxin provided that:

1. Unless directed otherwise by the HHS secretary, within 7 calendar days after identification, the select agent or toxin is transferred in accordance with 73.16 or destroyed on-site by a recognized sterilization or inactivation process.
2. The select agent or toxin is secured against theft, loss, or release during the period between identification of the select agent or toxin and transfer or destruction of such agent or toxin, and any theft loss or release of such agent or toxin is reported, and
3. The identification of the select agent or toxin is reported to the CDC or the Animal and Plant Health Inspection Service (APHIS) and to other appropriate authorities when required by federal state or local law.”¹⁹

The identification of certain agents in diagnostic specimens is of great concern to the CDC, and certain agents must be reported within 24 hours of identification. Exhibit 23-1 lists select agents and toxins with immediate reporting requirements, which is different from the reporting requirements for public health activities.

Additional variances are granted to the clinical laboratory to allow handling proficiency testing materials. As with diagnostic testing, the recipient of these materials must safeguard them from theft, loss, or release;

transfer or destroy the testing materials within 90 calendar days of receipt; and report identification of the agent or toxin within 90 calendar days. Both of these exceptions are important in that they allow exemption of clinical laboratories that may only handle such agents for short periods of time during diagnostics or proficiency testing periods. These laboratories, which are already registered and inspected by the College of American Pathologists, generally only handle small quantities of agent at any given time.

In addition to the specific allowances provided for clinical labs, there are guidelines for agents with general exclusions as follows:

- Any select agent or toxin that is in its naturally occurring environment provided it has not been intentionally introduced, cultivated, collected, or otherwise extracted from its natural source.
- Nonviable select agent organisms or nonfunctional toxins.
- Formalin-fixed tissues.
- Agents that have been granted exception as a result of their proven attenuations.

Attenuated virus and bacteria strains are listed on the CDC Web site. This is not a general exclusion for all “attenuated strains” of viruses or bacteria. If researchers want exemption from the provisions for a particular strain, a written request for exclusion with supporting scientific information on the nature of the attenuation must be submitted. Agents that have already received exclusion are listed in Table 23-1.

EXHIBIT 23-1

IMMEDIATE REPORTING REQUIREMENTS FOR SELECT AGENTS

DHHS Select Agents and Toxins	Overlap Select Agents and Toxins*
Ebola viruses	<i>Bacillus anthracis</i>
Lassa fever virus	Botulinum neurotoxins
Marburg virus	<i>Brucella melitensis</i>
South American hemorrhagic fever viruses (Junin, Machupo, Sabia, Flexal, Guanarito)	<i>Francisella tularensis</i>
Variola major virus (Smallpox virus)	Hendra virus
Variola minor (Alastrim)	Nipah virus
<i>Yersinia pestis</i>	Rift Valley fever virus
	Venezuelan equine encephalitis virus

DHHS: Department of Health and Human Services

* Biological agents and toxins that affect both humans and livestock are termed overlap agents.

TABLE 23-1
ATTENUATED STRAINS EXEMPTED FROM REGULATION

Agent	Qualifier	Effective Date of Exclusion
Avian influenza (highly pathogenic) virus	Recombinant vaccine reference strains—H5N1 and H5N3 subtypes	5/7/2003
<i>Bacillus anthracis</i>	Devoid of both plasmids pX01 ⁺ and pX02	2/27/2003
<i>Bacillus anthracis</i>	Devoid of pX02 (<i>Bacillus anthracis</i> Sterne, pX01 ⁺ ,pX02 ⁻)	2/27/2003
<i>Brucella abortus</i>	Strain RB51 (vaccine strain)	5/7/2003
<i>Brucella abortus</i>	Strain 19	6/12/2003
<i>Coccidioides posadasii</i>	Δ chs5 strain + Δ cts / Δ ard1 / Δ cts3 strain	10/14/2003
Conotoxin	Specially excluded are the class of sodium channel antagonist U-conotoxins, including GIIIA; the class of calcium channel antagonist w-conotoxins, including GVIA, GVII, MVIIA, MVIIC, and their analogs or synthetic derivatives; the class of NMDA-antagonist conantokins, including con-G, con-R, con-T and their analogs or synthetic derivatives; and the putative neurotensin agonist, contulakin-G and its synthetic derivatives	4/29/2003
<i>Coxiella burnetii</i>	Phase II, Nine Mile Strain, plaque purified clone 4	10/15/2003
Junin virus vaccine strain	Candid 1	2/7/2003
<i>Francisella tularensis</i> subspecies <i>novicida</i>	Utah 112 (ATCC 15482)	2/27/2003
<i>Francisella tularensis</i> subspecies <i>holoartica</i>	Live vaccine strains, includes NDBR 101 lots, TSI-GSD lots, and ATCC 29684	2/27/2003
<i>Francisella tularensis</i>	ATCC 6223, also known as strain B38	4/14/2003
Japanese encephalitis virus	SA 14-14-2	3/12/2003
Rift Valley fever virus	MP-12	3/16/2004
Venezuelan equine encephalitis virus	V3526 (virus vaccine candidate strain)	5/5/2003
Venezuelan equine encephalitis virus	TC-83	3/13/2003
<i>Yersinia pestis</i>	Strains that are pgm ⁻ due to a deletion of a 102-kb region of the chromosome termed the pgm locus. This includes strain EV or various substrains such as EV 76	3/14/2003
<i>Yersinia pestis</i>	Strains devoid of the 75 kb low-calcium response virulence plasmid such as Tjwidej S and CDC A1122	2/27/2003

ATCC: American Type Culture Collection
NMDA: N-methyl-D-aspartate

In addition to the exclusions for specific strains of viruses or bacteria, certain amounts of toxin are not considered to pose a significant risk to human health or agriculture. Therefore, the requirement for registration depends on the amount of toxin possessed. The

toxins listed in Table 23-2 (in the purified form or in combinations of pure and impure forms) are exempt from regulation if the aggregate amount under the control of a principal investigator does not, at any time, exceed the amount specified.

CENTERS FOR DISEASE CONTROL AND PREVENTION SAFEGUARDS

The CDC regulations require entities handling select agents to register and meet the following criteria:

- The entity must appoint an individual to represent it in its dealings with the CDC (this person is called the Responsible Official).

TABLE 23-2
REGULATED AMOUNTS OF TOXINS*

Toxin	Amount (mg)
Abrin	100
Botulinum neurotoxins	0.5
Conotoxins	100
Diacetoxyscirpenol	1,000
Ricin	100
Saxitoxin	100
Shiga-like ribosome-inactivating proteins	100
Staphylococcal enterotoxins	5
Tetrodotoxin	100

*Current information can be obtained from the Centers for Disease Control and Prevention Web site: <http://www.cdc.gov/od/sap/sap/exclusion.htm>.

- The entity must define what agents are being used and for what purposes.
- The entity must provide the names of persons having access to agents.
- The entity must implement plans for the bio-safety, security, and emergency management.
- Each person having access to those agents must have a security risk assessment. This assessment ensures that restricted persons (per Title 18 United States Code 175b)²⁶ are denied access to any select agent or toxin.

The Attorney General defines a restricted person²⁶ as someone who:

- is under indictment for a crime punishable by imprisonment for a term exceeding 1 year;
- 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 (as defined in section 102 of the Controlled Substances Act [21 United States Code 802]²⁷);
- is an alien illegally or unlawfully in the United States;
- has been adjudicated as a mental defect 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 which the Secretary of State has determined to have repeatedly provided support for acts of international terrorism (if the determination remains in effect); or
- has been discharged from the Armed Forces of the United States under dishonorable conditions.

Once an entity is registered, the CDC may inspect its facilities at any time to ensure that handling of select agents is in accordance with the regulation. If at any time an entity is not in substantial compliance, the certificate of registration may be revoked, and all research involving select agents must cease until the entity can again demonstrate compliance with the regulations. Oversight by the CDC/USDA and the requirement for registration of both facilities and personnel represent a significant step in increasing the security of select agents and toxins that have the capacity to adversely impact human health and agricultural activities.

US ARMY BIOSURETY

To adapt to the post-9/11 world, the US Army began to develop its own policies involving select agents and toxins. Although the CDC's policies focused on limiting access to select agent stocks, the Army Biosurety Program focused on the reliability of personnel who had been granted full access to select agents to ensure that they were qualified. The biosurety program is based on the military experience with surety programs for both nuclear and chemical weapons. The goals of the chemical and nuclear surety program are to ensure that operations with these hazardous materials are performed safely and securely. The intent of the biological surety program is the same, but its policies also consider the unique aspects of biological agents.

Review of the DoD biological research, development, test, and evaluation programs revealed a need to heighten security and implement more stringent procedures for controlling access to infectious agents.²⁸ In light of the

newly identified threats to the public health, emphasis and funding were provided to address these concerns. In addition to increased security and control measures, the Department of the Army (DA) inspector general advocated the immediate implementation of a biosurety program. Work on the program began quickly with a series of interim guidance messages (beginning in December 2001) to the DoD biological defense research community. The first message defined the general guidelines for the Army's Biosurety Program. The second and third messages addressed biological personnel reliability programs (BPRPs), contractor personnel, and facilities. The policies set forth in the interim messages were formalized with the implementation of the draft Army Regulation (AR) 50-X, *Army Biological Surety Program* (current version dated December 28, 2004),²⁹ which established the DA's corporate approach for the safe, secure, and authorized use of biological select agents and toxins (BSATs) and

identified the procedures for the BPRP. In January 2005 all agencies throughout the Army that handled select agents were directed to comply with the draft AR 50-X as of May 5, 2005. This compliance requirement represented a major effort in a comparatively short period of time for all Army agencies handling BSATs.

Surety Program Concepts

Biosurety is defined as the combination of four basic areas or pillars: (1) physical security, (2) biosafety, (3) agent accountability, and (4) personnel reliability.³⁰ The careful integration of these factors yields policies and procedures to mitigate the risks of conducting research with these agents. Physical security defines the actions that secure select agents and deny access to select agents for subversive purposes. Multiple layers of integrated levels of security can use a variety of means to detect intrusion and prevent theft or misuse of select agents. Biosafety, a term that has been used for many years and with various definitions, is best defined as the procedures used in the laboratory or facility to ensure that pathogenic microbes are safely handled. The procedures and facility design requirements defined in the *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 5th edition, are the standard for the safe handling of all infectious agents.³¹ Agent accountability means keeping accurate inventory records and establishing an audit to ensure that stocks are not missing. Personnel reliability is the final pillar in ensuring that those who are granted access to agents are stable, trustworthy, and competent to perform the tasks assigned to them. Although the screening procedures for the CDC's security risk assessment are designed to exclude restricted persons, the DoD policy uses methods to assess a person's reliability. Every person having access to select agents submits

to initial screenings followed by continuous health monitoring, random drug tests, and periodic evaluation by the supervisor to ensure that each employee maintains the highest standards of personal conduct. All of these programs contribute in important ways to the mission of biosurety. Table 23-3 shows the pillars and contributing factors of biosurety. The foundation for the pillars is training: continuous training in all of these areas helps ensure that personnel understand the mission and conduct research safely and securely.

Physical Security

One of the important factors in establishing a dynamic biosurety program is security. Developing a security plan begins by identifying areas containing select agents and toxins and limiting access to those areas. Typically this is done by establishing restricted areas and using automated access control systems. These systems provide detailed information, record access to restricted areas, and can even be tied into closed-circuit television cameras to allow positive identification of personnel before they are allowed entry. A combination of increasingly restrictive security measures can help to establish layers of security perimeters commensurate with the risk related to the agents used. For example, card readers can be used to limit and identify progress thorough corridors of restricted areas, whereas locks activated by personal identification number key pads allow entry into specific rooms. Laboratories containing high-risk agents, such as Ebola virus and botulinum neurotoxins, may have additional measures such as biometric readers and intrusion detection systems. Specific requirements for access may include clearly defined and visible markings on security badges. Everyone in the facility should be aware of the ways that restricted areas are

TABLE 23-3
PILLARS OF BIOSURETY AND PILLAR COMPONENTS

Physical Security	Safety	Personnel Reliability	Agent Accountability
Limited access to biological restricted areas	Safety training and mentorship	Background investigations	Agent inventory noting locations of agents
Internal and external monitoring	Risk management	Medical screening	Access to stocks limited
Intrusion detection systems	Environmental surveillance	Employment records screening	Accurate and current inventory of historical and working stocks
Random search and inspection	Occupational health screening	Urinalysis	Auditable records system

marked and who is allowed access to those areas to identify intruders. Persons who are allowed access to the restricted areas must have completed all training required for the safe conduct of laboratory procedures. Training should be evaluated through testing, or preferably, a period of mentorship within the containment. A mentorship program allows the trainee to experience the working conditions and ask questions under close supervision. The time required for mentorship periods depends on the level of experience of the person entering containment. The trainee should not be allowed unescorted access to a containment area until the trainer is satisfied that he or she can perform a variety of tasks safely and securely.

Biosafety

The guidelines regarding the safe handling of infectious agents and toxins and for laboratory design are defined in the *BMBL*.³¹ Before the establishment of these guidelines, it was not uncommon to have laboratory workers become infected with the agents that they were handling. Sulkin and Pike conducted a series of studies from 1949 until 1976 documenting and characterizing laboratory-acquired infections.³²⁻³⁵ These studies helped to identify problems with common laboratory procedures of the time (mouth pipetting, needle and syringe use, and generally poor techniques) that contributed to the rate of laboratory infection. Although many laboratory-acquired infections occurred with *Brucella*, *Salmonella*, *Francisella tularensis*, *Mycobacterium tuberculosis*, hepatitis virus, and Venezuelan equine encephalitis virus, less than 20% were associated with a "laboratory accident." Also, the infected laboratory workers were not considered a threat to the public health because of the low incidence of agent transmission to contacts.

In 1979 Pike concluded in a review that "the knowledge, the techniques and the equipment to prevent most laboratory-acquired infections are available."³⁶ However, it was not until 1984 that the CDC/National Institutes of Health published the first edition of the *BMBL*, which described combinations of standard and special microbiological practices, safety equipment, and facilities that constituted biosafety levels 1 through 4. This publication also defined for the first time which agents should be handled in which laboratory safety level. The implementation of these guidelines around the country has significantly reduced the occurrence of laboratory-acquired infections.³¹ Under 42 CFR Part 73, the entity is required to develop a biosafety plan that identifies the agents used and procedures for their safe handling and containment.¹⁹

The *BMBL* describes three areas necessary to establish containment: (1) laboratory practices and techniques, (2) safety equipment, and (3) facility design/construction. The combination of laboratory practices and primary and secondary barriers reduces the chances of exposure for laboratory personnel, other persons, and the outside environment to hazardous biological agents. In developing the laboratory-specific procedures and practices, it is important to integrate all aspects of these barrier protections. In addition to the procedures specific to their research protocol, all persons operating in containment laboratories should understand the operation of the safety equipment that serves as the primary barrier for containment. Examples of primary barriers include biological safety cabinets, glove boxes, safety centrifuge cups, or any other type of enclosure or engineering control that limits the worker's exposure to the agent. Secondary barriers are facility and design construction features that contribute to the worker's protection and also protect those outside of the laboratory from contact with or exposure to agents inside the containment facility. Examples of secondary barriers include physical separation of laboratory areas from areas that are accessible to the general public, hand-washing facilities in close proximity to exits, and specialized ventilation systems that provide directional flow of air and high-efficiency particulate air filtration prior to exhaust. Training for the performed protocols and laboratory-specific operations should be clearly defined and well documented. Depending on the risk of the activities being conducted in the containment laboratory, it is not sufficient to read a manual or receive a briefing to ensure proper training. In many cases, a method to assess the person's understanding and ability to perform these tasks should be used.

Biological Personnel Reliability Program

The purpose of the BPRP is to ensure that persons with access to potentially dangerous infectious agents and toxins are reliable. The program as defined in AR 50-X chapter 2 (Biological Surety) goes far beyond the CDC requirements for access to select agents. Although the CDC ensures that restricted persons do not have access to select agents, the BPRP further requires that persons with access to select agents are "mentally alert, mentally and emotionally stable, trustworthy, and physically competent." To this end, personnel undergo an initial screening process and then submit to continuous monitoring for the duration of their duties accessing select agents. This is the most detailed chapter in the biosurety regulation, and the program

requires dedicated efforts of many persons to ensure that it is executed fairly and coordinated with all of the screening partners.

The first step in the establishment of the program is to identify personnel who must be enrolled. AR 50-X identifies four categories of persons who must be enrolled:

1. personnel who have a legitimate need to handle or use BSATs;
2. personnel whose duties afford direct access to storage and work areas, storage containers, and equipment containing BSATs, including persons with responsibility for access control systems such that they could provide themselves direct access to storage and work areas, storage containers, and equipment containing BSATs;
3. armed security guards inside the facility, as identified in biological security guidance to be published by the Office of the Provost Marshall General; and
4. personnel authorized to escort visitors to areas containing BSATs.

The requirements for enrollment, therefore, are not restricted to researchers who use BSATs daily but may extend to people who receive shipments at the warehouse or service equipment within the containment laboratories. They are also not limited to a particular job series (Government Schedule [GS]) of a government employee but are instead related to the specific duties. For example, in one division, there may be two employees who are both GS-403 series DA civilians performing tasks as microbiologists, but only one microbiologist may be required to have access to select agents. Therefore, enrollment in the BPRP is required only for the employee who must access the agents. This requirement has created some difficulty in implementing the BPRP because persons with access to select agents may have little incentive to endure the rigorous screening process and continuous intrusive monitoring if they can perform similar research with nonselect agents or perform select agent research in a non-DoD laboratory. The possibility of losing talented and well-trained researchers to other facilities and non-DoD agencies with less stringent programs, a continuing concern, may impact the ability of the Defense Threat Reduction Agency to provide research personnel to combat biological agent use in the United States by terrorist organizations.

The initial screening process for enrollment requires a six-step process:

1. initial interview
2. personnel records review
3. personnel security investigation

4. medical evaluation
5. drug testing and
6. final review.

The order of steps in the process is left to the discretion of the activity; however, each step must occur and be fully documented.

Initial Interview

The process begins with the initial interview conducted by the certifying official (CO). The CO is the gatekeeper for access to select agents and toxins, ensuring that persons requesting access have met all of the qualifying conditions. Typically, the CO supervises the worker or is otherwise in the supervisory chain. During the initial interview, the candidate grants consent for the screening and 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 suitability. If exceptional extenuating circumstances exist, reviewing officials may request an exception for the enrollment of the individual through their command channels. The following are mandatory disqualifying factors:

- Diagnosis as currently alcohol dependent based on a determination by an appropriate medical authority.
- Drug abuse in the circumstances listed below:
 - o Individuals who have abused drugs in the 5 years before the initial BPRP interview. Isolated episodes of abuse of another person's prescribed drug will be evaluated.
 - o Individuals who have ever illegally trafficked in illegal or controlled drugs.
 - o Individuals who have abused drugs while enrolled in the BPRP, including abuse of another individual's prescribed drugs.
- Inability to meet safety requirements, such as the inability to correctly wear personal protective equipment required for the assigned position, other than temporary medical conditions. Questions regarding the duration of medical conditions will be referred to a competent medical authority.

The initial interview also determines whether any instances of potentially disqualifying activities exist. These are activities that the CO must consider when evaluating a person's reliability for access to BSATs. Potentially disqualifying factors are much broader and

are evaluated by the CO to establish a full picture of the person's character. The following excerpt from AR 50-X describes potentially disqualifying factors:

a. Alcohol-related incidents/abusing alcohol.

(1) Certifying officials will evaluate the circumstances of alcohol-related incidents that occurred in the 5 years before the initial interview and request a medical evaluation. An individual diagnosed through such medical evaluation as currently alcohol dependent will be disqualified per paragraph 2-7a, AR 50-X. Individuals diagnosed as abusing alcohol will be handled per paragraph (2) below. For an individual not diagnosed as a current alcohol dependent/abusing alcohol, including those individuals identified as recovering alcoholics, the CO will determine reliability based on results of the investigation, the medical evaluation, and any extenuating or mitigating circumstances (such as successful completion of a rehabilitation program). The CO will then qualify or disqualify the individual from the BPRP, as he or she deems appropriate.

(2) Individuals diagnosed as abusing alcohol but who are not alcohol dependent, shall at a minimum be suspended from BPRP processing pending completion of the rehabilitation program or treatment regimen prescribed by the medical authority. Before the individual is certified into the program, the CO will assess whether the individual has displayed positive changes in job reliability and lifestyle, and whether the individual has a favorable medical prognosis from the medical authority. Failure to satisfactorily meet these requirements shall result in disqualification.

b. Drug abuse.

(1) In situations not otherwise addressed in paragraph 2-7b, a CO may qualify or disqualify an individual who has abused drugs more than 5 years before the initial BPRP screening, or have isolated episodes of abuse of another's prescription drugs within 15 years of initial BPRP screening. In deciding whether to disqualify individuals in these cases, the CO will request medical evaluation and may consider extenuating or mitigating circumstances. To qualify the individual for the BPRP, the CO's memorandum of the potentially disqualifying information (PDI) must include an approval signed by the reviewing official. Ex-

amples of potential extenuating or mitigating circumstances include, but are not limited to:

- (a) Successful completion of a drug rehabilitation program.
- (b) Isolated experimental drug abuse.
- (c) Age at the time of the drug abuse ("youthful indiscretion").

(2) Certifying officials may qualify individuals whose isolated episodes of abuse of another's prescription drugs occurred 15 or more years before the initial BPRP screening without medical review or additional reviewing official approval. Certifying officials will consider such abuse in conjunction with other PDI in determining reliability of the individual.

c. Medical condition.

Any significant mental or physical medical condition substantiated medically and considered by the CO to be prejudicial to reliable performance of BPRP duties may be considered as grounds for disqualification from the BPRP. In addition, the medical authority will evaluate individuals and make a recommendation to the CO on their suitability for duty in the BPRP in the following circumstances:

- (1) Individuals currently under treatment with hypnotherapy.
- (2) Individuals that have attempted or threatened suicide before entry into the BPRP.
- (3) Individuals that have attempted or threatened suicide while enrolled in the BPRP. To qualify such an individual for the BPRP, the CO's memorandum of the PDI (paragraph 2-15a) must include an approval signed by the reviewing official.

d. Inappropriate attitude or behavior.²⁹

In determining reliability, the CO must conduct a careful and balanced evaluation of all aspects of an individual. Specific factors to consider include, but are not limited to:

- negligence or delinquency in performance of duty;
- conviction of, or involvement in, a serious incident indicating a contemptuous attitude toward the law, regulations, or other duly constituted authority. Serious incidents include, but are not limited to, assault, sexual misconduct, financial irresponsibility, contempt of court, making false official statements, habitual traffic offenses, and child or spouse abuse;

- poor attitude or lack of motivation. Poor attitude can include arrogance, inflexibility, suspiciousness, hostility, flippancy toward BPRP responsibilities, and extreme moods or mood swings;
- aberrant behavior such as impulsiveness or threats toward other individuals; and
- attempting to conceal PDI from CO through false or misleading statements.

Personnel Records Review

Once the CO has completed the initial interview and found the candidate to be suitable for enrollment, human resources personnel screen the candidate's official employment or service history records to identify any problematic areas of job performance. Anything that may indicate unsatisfactory employment history or dereliction of duty should be reported to the CO for consideration as PDI. Job applications, enlistment contracts, and any other record available to the personnel screener should be reviewed for PDI.

Personnel Security Investigation

Personnel security investigation dossiers are screened by the personnel security specialist for PDI. Personnel scheduled for initial assignment to BPRP positions must have the appropriate and favorably adjudicated personnel security investigation completed within the 5 years preceding certification to the BPRP. The minimum personnel security investigation required for military and contractor employees is the National Agency Check, Local Agency Check, and Credit Check. The minimum personnel security investigation for civilian employees is the Access National Agency Check with Written Inquiries; a National Agency Check, Local Agency Check, and Credit Check is also acceptable for civilian employees. Higher level investigations are acceptable provided they have been completed within the past 5 years.

Medical Evaluation

The medical evaluation ensures that the person being certified is physically, mentally, and emotionally stable; competent; alert; and dependable. A competent medical authority is charged with conducting a review of military health records and civilian occupational health records to assess the individual's health. If the medical record is not sufficiently complete for the medical authority to provide a recommendation to the CO, then a physical examination must be conducted. 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 personal protective equipment, or impaired ability to perform the physical requirements of the BPRP position, as substantiated by the medical authority to the CO. Medical PDI is reported to the CO with the recommendations regarding the person's fitness for assignment to these duties. The competent medical authority should again consider these factors when determining the scope and duties of personnel within containment research laboratories.

Drug Testing

The next step in the screening is to conduct a urinalysis. This screening must be done within a 6-month window of the final review and before being certified as reliable and suitable for assignment to duties requiring handling of BSATs. In most cases, military personnel are already performing a command-directed urinalysis. If they have had a negative test reported within 6 months, there is no additional testing required. However, if they have not been tested under the command randomized program within the past 6 months, arrangements must be made with the commander for a specially coded BPRP urinalysis. For DA civilians, the majority of research personnel have never been part of a testing designated pool. This testing must be completed according to DHHS standards as published in the Mandatory Guidelines for Federal Workplace Drug Testing programs. For most DA civilians, this will require that their position be a test-designated position, which then allows the Army to require urine drug testing. AR 600-85 is the Army regulation governing this program under the direction of the Army Substance Abuse program offices at every installation. This regulation is being revised to include biological BPRPs in the same sensitive position category as the nuclear and chemical BPRPs. The testing of contractor employees is the responsibility of the contractor; however, the biosurety officer must provide the oversight to the contractor to ensure that testing is being performed properly.

Final Review

After the candidate has completed all phases of the screening, the CO conducts a final review to inform the individual of any PDI disclosed to the CO during the screening process. The review provides an opportunity for discussing the circumstances in which the potentially disqualifying events took place before the CO's decision on the candidate's suitability for the program. At the end of the interview, the CO should inform the

candidates if they are suitable for the program and discuss the expectations for continuous monitoring. AR 50-X lists eight areas that must be briefed to the individual during the final interview:

1. The individual has been found suitable for the BPRP.
2. The duties and responsibilities of the individual's BPRP position.
3. Any hazards associated with the individual's assigned BPRP duties.
4. The current threat and physical security and operational security procedures used to counter this threat.
5. Each person's obligations under the continuing evaluation aspects of the BPRP.
6. A review of the disqualifying factors.
7. The use of all prescription drugs must be under the supervision of a healthcare provider. While in the BPRP, any use of any drugs prescribed for another person is considered drug abuse and will result in immediate disqualification.
8. Required training before the individual begins BPRP duties.

At the end of the interview, the CO and the candidate sign DA Form 3180 indicating their understanding of the programs and their willingness to comply with the requirements. The person is then "certified" and subject to continuous monitoring.

Continuous Monitoring

During the continuous monitoring phase, BPRP personnel are required to self-report any changes in their status and observations of other BPRP employees. Any changes in medical status should be evaluated by the competent medical authority. Periodic reinvestigations should be conducted every 5 years, and urine drug testing should be conducted at least once every 12 months for military personnel and randomly for DA civilians and contractors. Medical monitoring and routine physical examinations should be conducted periodically depending on the type of containment work being performed.

Agent Accountability

Agent accountability in the research field presents a new challenge. Microbiological agents are replicating organisms; thus, the accounting for each and every microbe is meaningless over time. As an example, the recorded transfer showing the receipt of 1 mL of any

replicating agent and the subsequent shipment of 1 mL to a second researcher does not mean that the first researcher no longer holds stocks of that agent. The recipient researcher can use the original 1 mL of agent to create 50 more 1-mL vials of the same agent. In this sense, every researcher has the capability to be a small-scale production facility, which makes for a dynamic inventory environment requiring clear guidelines and meaningful documentation requirements to ensure a current and accurate record.

Title 42 CFR 73 states that an "entity required to register under this part must maintain complete records relating to the activities covered by this part" and specifies the data points that must be captured.

Such records must include: (1) accurate, current inventory for each select agent (including viral genetic elements, recombinant nucleic acids, and recombinant organisms) held in long-term storage (placement in a system designed to maintain viability for future use, such as a freezer or lyophilized materials), including: (i) the name and characteristics (eg, strain designation, GenBank accession number, etc); (ii) the quantity acquired from another individual or entity (eg, containers, vials, tubes, etc), date of acquisition, and the source; (iii) where stored (eg, building, room, and freezer); (iv) when moved from storage and by whom and when returned to storage and by whom; (v) the select agent used and purpose of use; (vi) records created under § 73.16 and 9 CFR 121.16 (transfers); (vii) for intra-entity transfers (sender and the recipient are covered by the same certificate of registration), the select agent, the quantity transferred, the date of transfer, the sender, and the recipient; and (viii) records created under § 73.19 and 9 CFR Part 121.19 (notification of theft, loss, or release). (2) Accurate, current inventory for each toxin held, including: (i) the name and characteristics; (ii) the quantity acquired from another individual or entity (eg, containers, vials, tubes, etc), date of acquisition, and the source; (iii) the initial and current quantity amount (eg, milligrams, milliliters, grams, etc); (iv) the toxin used and purpose of use, quantity, date(s) of the use and by whom; (v) where stored (eg, building, room, and freezer); (vi) when moved from storage and by whom and when returned to storage and by whom including quantity amount.¹⁹

With these criteria, it is possible to determine who accesses select agents, as well as when and where they were accessed. Although this may be rather easily accomplished in a facility where a limited number of persons has access to agents and uses them infrequently, it is more challenging in facilities with multiple storage sites, research areas, and principal investigators directing the activities of multiple investigators in shared laboratory suites.

AR 50-X gives the minimum requirements for site-specific standing operating procedures that address each entity's activities. The intent of AR 50-X is to have a clear audit trail of custody from receipt to destruction or transfer. Although laboratory notebooks may capture some aspects of the data, they do not provide a system that is sufficiently dynamic to meet the need for documentation and management of research stocks. Automation of these records will allow the retrieval of the information that is required for both researchers and those ensuring that the research is compliant with regulatory guidelines.

The draft AR 50-X limits entities that the Army can

transfer select agents to without further oversight. Requests to transfer Army BSATs must be approved by the assistant to the secretary of defense for nuclear and chemical and biological defense programs. Most requests to transfer must identify recipient information, name and quantity of the agent to be provided, purpose for which the BSATs will be used, and the rationale for providing the agent. In approving the request, the assistant to the secretary of defense may require conformance to biosurety measures for the recipient that are beyond those of the DHHS, USDA, and APHIS federal regulations.

SUMMARY

The programs securing select agents currently being implemented are detailed and complex. However, the intent of these programs remains simple: to keep biological agents that can cause catastrophic impact to humans, animals, and plants out of the hands of those who wish to use them for malicious intent. Although biological agents that remain in the environment often do not pose a threat to large populations, the quantities of agents produced and purified for research purposes could be used to incite panic, cause pandemic disease, and disrupt the industrial base of the United States. The procedures implemented by the DHHS and APHIS represent a significant step in securing these agents throughout the country. These agencies require entities to register and declare the agents in their possession, to ensure that the agents are handled under the appropriate safety and security controls, and to ensure that all persons who have ac-

cess to select agents have undergone a security risk assessment. These agencies also require that an entity develop emergency response plans, rehearse these plans with local and federal response teams, and keep accurate and current inventory records so that any loss or theft could be rapidly addressed. In addition to screening for restricted persons, the Army has taken a further step to ensure that personnel with access to select agents are trustworthy, physically able, mentally stable, and well trained for conducting research with these agents. Not only will persons who work with the agents within DoD institutes meet these standards, but also those with whom DoD shares research tools may be held to this higher standard. The immensity of this task cannot be overstated, but it is an important step in maintaining the public trust in performance of the vital research leading to effective countermeasures against biological threat agents.

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Chapter 24

ETHICAL AND LEGAL DILEMMAS IN BIODEFENSE RESEARCH

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INTRODUCTION

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The Animal Efficacy Rule
BioShield Act of 2004
The Turner Bill
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SUMMARY

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INTRODUCTION

The anthrax attacks of October 2001 made the nation acutely aware of not just the possibility of a large-scale biological weapons attack on US soil, but also has brought to the forefront concerns over the proper measures to be implemented to prepare for such biological warfare scenarios. It is evident that drugs and vaccines may be needed immediately to respond appropriately to emergency or battle situations. Government regulatory agencies, the pharmaceutical industry, and the armed services must work together more effectively so that vaccines and 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 accustomed to responding 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, understandably, the primary objectives of pharmaceutical companies, so drugs are more likely to be developed for common rather than rare diseases. For such naturally occurring, often relatively common diseases, many potential test subjects are ready and willing 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 humans have learned it is not safe to settle, or they occur in sporadic, small epidemics that kill everyone affected and fail to spread. In any case, there are rarely enough “naturally” occurring disease outbreaks of this kind to conduct clinical trials yielding substantial evidence of human clinical efficacy.

Over the past 60 years the conditions that must be met in order 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, Food and Drug Administration (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 takes a long time. Although some disease agents cause sporadic epidemics, others only infect individuals randomly

when they happen upon a reservoir of contagion. Biowarfare attacks involving these uncommon agents would likely affect many people suddenly, permitting neither the opportunity to enroll enough subjects in a study nor the time for observation. Although FDA restrictions are meant to protect the public from possible harm, delaying use of potentially beneficial products until outcomes are known can be detrimental 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 to suffice if the circumstances cannot permit valid human clinical 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 not approved for marketing are classified as investigational new drugs (INDs). FDA regulations specify what is necessary to change from the latter status to the former.

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. But in the military context, FDA regulations pose three significant legal hurdles to the military’s ethical responsibility to protect military personnel. First, because diseases that are potential weapons, such as Ebola or Rift Valley fever, are both rare in nature 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 INDs (as opposed to single use instances) in emergency life-threatening situations, is illegal. Third, it is illegal to systematically use licensed drugs in large numbers of persons for uses other than those indicated on the label. Ultimately, however, researchers must find ways to circumvent these limitations so that the FDA and Department of Defense (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 medicine. When the letter of the law is applied, the interests of military personnel may be lost in the shuffle, leaving 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 at hand. Some way to bridge the two horns of this dilemma is needed; in particular, there must be a legal way to make protective drugs and vaccines available when the normally required clinical trials cannot be carried out.

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 219.109.¹ 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 biodefenses and protections. 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.² 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

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 might pursue in relation to the issues outlined. Some of the legislated solutions recently proposed or implemented will also be included.

placed into crowded camps, mingling with local civilian populations, which expanded variola transmission even further into vulnerable populations.³ 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, his actions were criticized by a public unfamiliar with the stakes or conditions weighing on this choice (Figure 24-1).



Fig. 24-1. 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.

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.⁴ 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.⁵⁻⁷

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 state-

ments could be considered "personal service contracts" (Figure 24-2). 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 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.⁸ In 1932 the secretary of the Navy granted permission for experiments with divers, provided they were "informed volunteers."⁹

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. The attempt was thwarted by vigilant scientists, 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."¹⁰ 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:

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, volunteers only should be utilized as subjects, and these only after the risks have been fully explained and after signed statements have been

Figure 24-2 continued

b

El que suscribe, *Antonio Benigno*
mayor de veinte y cinco años de edad, natural de *Cercada*
provincia de *Coruña* hijo de *Manuel Benigno*
y de *Josefa Castro* hace constar por la presente que, estando y
ejerciendo su propia y libre y voluntaria, consiente en someterse a los
experimentos que con el objeto de determinar las vias de propagacion de
la fiebre amarilla, hace en su persona la Comision que para ese efecto se
nombrara el Secretario de la Guerra de los Estados Unidos; que de su consentimiento para que se lleven a cabo dichos experimentos, por las razones y
con las condiciones que abajo se expresan.

El infrascripto comprende perfectamente bien que en el caso de contra-
cerse en él la fiebre amarilla, se va a padecer su vida hasta cierto punto
pero siéndole completamente imposible evitar el contacto durante su permanen-
cia en esta Isla, creeiere exponer la posibilidad de contraerla ex-ociso,
con la seguridad de que se le recibirá de la Comision y/o sus agencias, los
cuidados mas profundos y la asistencia médica mas esmerada.

Cuando consintiere que se terminen estos experimentos, antes de transcur-
rir los meses de este mes, el infrascripto no se recibirá la suma de \$ 100...
oro americano y que caso de padecerse en él la fiebre amarilla, en cualquier
etapa durante su permanencia en este Campamento, recibirá además de dicha
cantidad, otra suma de \$100. --oro americano, despues de su curacion y en
caso de su fallecimiento por motivo de esa enfermedad, la Comision entregará
dicha cantidad, (o equivalentes desde americano,) a la persona que él su testigo
designará el infrascripto.

El infrascripto se compromete a no salir de los límites de este Campa-
mento durante el periodo de los experimentos y renunciará todo derecho a los
beneficios de este contrato si rompiere este compromiso.

Y para su constancia firma este por duplicado, en el Campamento experi-
mental, cerca de los Cuarteles, Guca, el día *26* de *Noviembre*
de mil novecientos.

El interesado,
Antonio Benigno

De conformidad, la Comision.
Walter Reed
Waf. Samp. USA.

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.

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.¹¹

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 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—the quick, successful development 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 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 24-1).

The DoD adopted all of the elements of the Nuremberg Code verbatim and added a prisoner-of-war provision.¹² 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*,¹³ which regulated Army research until 1983.

In 1952 the Armed Forces Medical Policy Council noted that nonpathogenic biological warfare simulations conducted at Fort Detrick 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.^{14,15} 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 24-3 and 24-4). 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 became the US Army Medical Research Institute of Infectious Diseases (USAMRIID).

In 1955 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 diseases such as Q fever and tularemia to understand how these illnesses affected the body and to determine indices of human vulnerability that might 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

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.

Exhibit 24-1. 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.

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 24-5)

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



Fig. 24-3. 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.

ask questions about risks, potential benefits, and the 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 participants that the research was scientifically valid and potentially dangerous, and that any harm to the participants would be minimized.

Approximately 150 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, 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.

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 1964.¹⁶

Congress enacted the National Research Act of 1974 because federally funded researchers violated human subjects’ rights, most famously in the Tuskegee syphilis experiments. This act immediately imposed rules for the protection of human subjects involved in research, requiring informed consent from subjects and review of research by institutional review boards. The act created the National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research, which



Fig. 24-4. 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.

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CONSENT STATEMENT

Regraded *Unclassified* by
 authority of *US Dept of Health, Education & Welfare*
 by *M. M. Hanley* 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 Signature]

WITNESS: ASN [Redacted]

[Redacted Name] Date **JUN 29 1955**

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Fig. 24-5. 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.

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 1983.¹³

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,¹⁷⁻¹⁹ and they remain appreciated as a practical approach

IMPACT OF REGULATING AGENCIES ON STRATEGIC RESEARCH

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 Cosmetics 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.²⁰

In 1962 Congress passed the FDA Kefauver-Harris Drug Amendments, which effectively launched the modern US drug regulatory system. These amendments stipulated an intense premarketing approval

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.

Success in incorporating ethical principles into human subjects research in the military in the early and mid 20th century was complemented by military researchers' numerous achievements in vaccine development with a variety of infections, including yellow fever (1900), typhoid fever (1911), pneumonia (1945), hepatitis A (1945), influenza (1957), rubella (1961), adenovirus (1952–1969), and meningitis (1966).³

system, giving the 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 INDs, 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. (Of course, 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 and 1974]¹⁴ 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. The most recent MOU provided the FDA an assurance that the DoD would conduct clinical testing of biologics, categorized as INDs, 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).²¹ The MOU states that the DoD will meet these requirements without jeopardizing responsibilities related to its mission of protecting national interests and safety.

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 battle zones.

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 through use of military force. 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 vaccination against credible threats. Vaccinations include licensed anthrax and smallpox vaccines and unlicensed vaccines for 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,²² 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.

Investigational New Drug Status of Vaccines

FDA considers any administration of an IND to a human to constitute research and authorizes the administration of an investigational product only in the context of a clinical research trial. Because the therapeutic benefit of the IND is unknown, FDA also requires informed consent. Administration of an IND 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. Collecting data from and recordkeeping for 100,000 soldiers would take exponentially longer than merely administering an unlicensed vaccine for treatment or prevention purposes. The consenting process alone for 100,000 individuals receiving an IND 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 tasks. 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 occurred during the Persian Gulf War. FDA regulations governing storage and distribution of INDs (21 CFR 312.57 and 59)²¹ are rigid and restrictive, which would render 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 INDs, including the vaccines in question, for research purposes. However, the situation in war is not a research situation. Giving these products to military personnel before engagement in war for purposes of thwarting the onset of some horrific disease constitutes a treatment application of the product, not research. No benefit is believed to accrue to an individual receiving an IND. Thus, administration of IND vaccinations 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 vaccine, even if there is limited evidence that the vaccine 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 INDs. Consequently, subjects have the right to decide whether they will receive the IND, and soldiers understand that they cannot be required to take IND 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 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. Specifically, one of the rights that military personnel forsake is the discretionary authority over their medical treatment. 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 animal efficacy rule or by BioShield emergency use authorization, 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 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 INDs.

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. Despite repeated high visibility studies conducted by the Institute of Medicine of the National Academies of Science, no evidence of causal relation has been shown between these symptoms and receipt of vaccine. 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 a vaccine involved the anthrax vaccination, which was an FDA-licensed product 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.²³⁻²⁵ 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. The DoD had an obligation to meet this extra threat, for the health of its soldiers, and for the benefit of the military mission. To meet this threat in as ethical a manner as possible, subject matter experts weighed in on risks and benefits of the use of the vaccine, and discussions between the DoD and FDA were held. That there may have been ill effects from the vaccine is an unintended consequence of the situation, the facts of which could not have been known beforehand, and which 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

Human Subjects Protections Regulations are Incompatible with Department of Defense Deployments

The immediacy of war preparations works 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 force health protection (FHP) measures cannot be “voluntary.” The voluntary nature of FDA-regulated research could

undercut the effectiveness of FHP measures, which rely on universal compliance for their efficacy. FHP measures, which are necessary for success 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. 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 INDs can undermine public trust and military morale. FDA requirement for informed consent for receipt of an IND is premised on the idea that administration of an IND is for research purposes, and the safety and efficacy of the drug are unknown. 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.

OPTIONS FOR FULFILLING MISSION AND ETHICAL RESPONSIBILITIES TO MILITARY PERSONNEL

Option 1: Continue to Use Investigational New Drug Products Without Full Compliance

The DoD can continue to use IND products, even though full compliance will not be achieved. GCP conflicts 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 gathered on these INDs during wartime are to be used for increasing product knowledge, then GCP restrictions should be relaxed for wartime military use. These changes would permit the DoD to contribute to research by adding to the data gathered before bringing INDs to market. DoD can choose to move forward with a particular IND 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

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 expediciencies of real-time deployment and the exigencies of warfare, making it difficult for the DoD to meet FDA requirements. Protocol violations 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.

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 IND requirements that cannot be practicably met in specific cases. Finally, the DoD and FDA could negotiate and agree to an updated MOU that permits the exemption of certain products for contingency use in protecting or treating soldiers.

Problems

The potential for DoD abuse of such power, or even the perception of abuse of such powers, will always be present.

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)^{21,26,27} are not absolute. If informed consent is unfeasible or contrary to the best interests of recipients (21 CFR 50),²¹ 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.²⁸ 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 institutional review board 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 recent power to waive informed consent requirements for military personnel, including conflicts and limitations posed by Title 10 USC Section 980 (10 USC 980),²⁹ AR 70-25.¹³ 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 subjects is obtained in advance.³⁰

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.

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.”¹³ Thus, the Army's own regulations can be interpreted to conflict with a presidential waiver of consent, and if soldiers cannot be compelled to receive vaccines or drugs intended to fight diseases, the presidential waiver fails to accomplish its intent.

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, 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, contains a provision to “fast track” certain products under the Federal Drug Act, including 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.”³⁰

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, will apply 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 myriad of 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. 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.

BioShield Act of 2004

Perhaps the most promising solution to the current impasse is the BioShield Act of 2004, which President George W Bush outlined in his 2003 State of the Union address as a key legislative priority for his administration. Project BioShield is 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 creates permanent funding for the procurement of medical countermeasures and gives 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 newly created 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, Mich) was approved by Health and Human Services Secretary Tommy G Thompson on January 14, 2005, authorizing its emergency use.

However, Project BioShield contains a provision that still conflicts with DoD discretionary authority over medical treatment for military personnel, continuing to require voluntary willingness to receive a vaccine or other treatment approved under the category of "emergency use." Although the language in the legislation refers specifically to "civilians," how this requirement will play out in the military setting, especially in wartime, is unclear. For maximum military effectiveness, a further stipulation in the legislation is required that the voluntary acceptance of treatment be waived in emergency situations, presumably on authority of the president of the United States with expert opinion from ethicists, legal scholars, and scientists. Additionally, there is no profit motive for private companies to engage in the research that this legislation aims to foster, and indemnification concerns also exist. There is no guarantee of efficacy of the theoretical drug or vaccine, and accountability measures should be created if the legislation is going to achieve its intended results.

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 emergency use authorization 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 but had not been approved for use against chemical warfare agents.

Biodefense and Pandemic Vaccine and Drug Development Act of 2005

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.

SUMMARY

This chapter has provided a view of the history of ethically conducted human subjects 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 biochemical weapons 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.

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Chapter 25

EMERGING INFECTIOUS DISEASES AND FUTURE THREATS

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INTRODUCTION

EMERGING BACTERIAL DISEASES

- Waterborne Diseases
- Foodborne Diseases
- Tick-borne Diseases
- Emerging Antibiotic Resistance

EMERGING VIRAL DISEASES

- Avian Influenza and the Threat of Pandemic Influenza
- Severe Acute Respiratory Syndrome
- Emerging Paramyxoviruses
- Emerging Arthropod-borne Viruses: Dengue and West Nile Viruses

GENETICALLY ENGINEERED THREATS

SUMMARY

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INTRODUCTION

Emerging infectious diseases, as defined in the landmark 1992 report by the Institute of Medicine, are diseases whose incidence has increased within the past 20 years or whose incidence threatens to increase in the near future.¹ Even though some “emerging” diseases have now been recognized for over 20 years (eg, acquired immunodeficiency syndrome [AIDS], Lyme disease, Legionnaire’s disease), their importance has not diminished, and the factors associated with their emergence are still relevant. Emerging infections include diseases caused by new agents (or newly described agents) and reemerging pathogens (those whose incidence had previously declined but now is increasing). This definition also includes organisms that are developing antimicrobial resistance and established diseases with a recently discovered infectious origin.

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 infections such as AIDS); changes in land use (eg, deforestation and reforestation), climate, and weather; international travel and commerce; and microbial or vector adaptation and change (mutations which result in drug or pesticide resistance).¹ 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 issues.

The numerous examples of “new” infections originating from animal species (ie, zoonoses) suggest that the zoonotic pool is an important and potentially rich source of emerging diseases.² Although classify-

ing AIDS as a zoonotic disease is controversial,³ it is now clear that both human immunodeficiency virus [HIV]-1 and HIV-2 had zoonotic origins.^{4,6} In addition, as shown by 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 severe acute respiratory syndrome (SARS) coronavirus.⁷

Many of the viruses or bacteria that may be potential bioweapons are considered emerging pathogens. In particular, some of these agents have appeared in new geographical locations where they have not previously been seen; for example, monkeypox suddenly occurred in the US Midwest in 2003, and the largest recorded outbreak of Marburg hemorrhagic fever occurred in Angola in 2005. Sometimes the specific use of a pathogen in an act of bioterrorism can cause the pathogen to be classified as an emerging or reemerging disease agent, as what happened with *Bacillus anthracis* during the 2001 anthrax attacks in the United States. Through increasingly easy molecular biology techniques, completely new organisms (or significantly modified existing organisms) can now be made in the laboratory. The use of these methods is mostly beneficial and necessary for modern biomedical research to proceed. However, the same methods and techniques can be used for destructive purposes and, along with naturally occurring emerging infections, represent significant future threats to both military and civilian populations.

EMERGING BACTERIAL DISEASES

Waterborne Diseases

Emerging waterborne diseases constitute a major health hazard in both developing and developed countries. In 2001 and 2002, 31 disease outbreaks associated with contaminated drinking water were reported in the United States, resulting in 1,020 ill people and 7 deaths.⁸ During this same time, over 2,500 cases of illness and 8 deaths nationally were associated with recreational waterborne diseases.⁹ Bacterial pathogens associated with drinking water disease outbreaks included *Legionella* species, *Escherichia coli* O157:H7, and *Campylobacter jejuni* (one outbreak each), and one outbreak involving infection with two different bacteria: *C jejuni* and *Yersinia enterocolitica*.⁸ Bacterial

pathogens responsible for gastroenteritis outbreaks associated with recreational water exposure included *E coli* O157:H7 (four outbreaks) and *Shigella sonnei* (two outbreaks). Twenty dermatitis outbreaks associated with spa or pool use were attributed to *Pseudomonas*, primarily *P aeruginosa*.⁹

Vibrio cholerae and Cholera

Accounts of cholera date to Hippocrates.¹⁰ 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.¹¹ This

event underscores the potential for cholera to cause widespread illness where water is not disinfected with a modern bactericide such as chlorine.¹¹

In 1991, after almost a century without cholera, outbreaks in Latin America resulted in about 400,000 cases of cholera and over 4,000 deaths.¹² Off the Peruvian coast, a significant correlation between cholera incidence and elevated sea surface temperature occurred between 1997 and 2000, which included the 1997–1998 El Niño event.¹³ Some people believe that the eighth worldwide pandemic began in 1992 with the emergence and spread of a new epidemic-causing strain (see below).¹⁴ During 2003, 45 countries reported a total of about 112,000 cases and almost 1,900 deaths from cholera.¹⁵ Paradoxically, cholera cases in the United States have decreased to about 10 cases per year during 1995 through 2000. Most of these cases were associated with either travel or 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 the minimum infective dose able to survive stomach acid.¹⁶

Before 1992, all 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.^{17,18} Cholera vaccines have had mixed success. Historically, live attenuated vaccines are more effective than killed whole-cell vaccines.¹⁹ No licensed cholera vaccines are available in the United States.

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.

Other Vibrios

In recent years, some noncholera vibrios have acquired increasing importance because of their association with human disease. Over 70 members are in the family *Vibrionaceae*, 12 of which have been isolated

from human clinical specimens and apparently are pathogenic for humans.²⁰ *Vibrio* species are primarily aquatic and are very common in marine and estuarine environments and on the surface and in the intestinal tracts of marine animals. *V parahaemolyticus* 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 parahaemolyticus* infections. *V vulnificus*, the most common source of vibrio infections in the United States, results in gastrointestinal symptoms similar to those of *V parahaemolyticus* and may also lead to ulcerative skin infections if open wounds are exposed to contaminated water. Septicemia can also occur in infected persons 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*



Fig. 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.

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.²⁰

Legionella Species

Legionnaire's 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.²¹ The cause of the outbreak remained a mystery for 6 months until the discovery by Joseph McDade, a Centers for Disease Control and Prevention microbiologist, of a few gram-negative bacilli, subsequently named *Legionella pneumophila*,²² in a gram stain of tissue from a guinea pig inoculated with lung tissue of a patient who died from the disease.²³ Using the indirect immunofluorescence assay, McDade showed that the sera of patients from the convention mounted an antibody response against the newly isolated bacterium,²⁴ marking the discovery of a whole new family of pathogenic bacteria. Retrospective analysis, however, has shown that outbreaks of acute respiratory disease from as far back as 1957 can be attributed to *L. pneumophila*.^{24,25} The earliest recorded isolate of a *Legionella* species was recovered by Hugh Tatlock in 1943 during an outbreak of Fort Bragg fever.^{26,27}

Legionnaire's 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. Legionnaire's disease has been associated with various water sources where bacterial growth is permitted, including cooling towers,²⁸ whirlpool spas,²⁹ and grocery store mist machines.²⁹ The association between a potable shower and nosocomial legionellosis was demonstrated 25 years ago.³⁰ The most common source of legionellosis in hospitals is from the hot water system,³¹ and sustained transmission of Legionnaire's disease in the hospital can be difficult to control.³² Community-acquired legionellosis is thought to account for most infections.³³ A recent Italian survey of household hot water systems 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.³⁴

Legionnaire's disease is an acute bacterial illness. Patients initially present 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.³⁵⁻³⁷ Attack rates after exposure to an aerosol-generating source are exceptionally high, often in the range of 50% to 80%. 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.³⁸

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 48 named species of *Legionella*, with at least 20 known to cause human infections.³⁹ 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.^{40,41} Three LLAP strains are now named *Legionella* species,⁴² and one of them, LLAP-3, was first isolated from the sputum of a patient with pneumonia by coculture with amoebae and is considered a human pathogen.⁴³

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.⁴⁴ Among the bacterial pathogens estimated to cause the greatest number of US foodborne illnesses are *Campylobacter*, *Salmonella*, *Shigella*, *Clostridium*, and *Staphylococcus* species.⁴⁴ Emerging bacterial illnesses include *E. coli* O157:H7 and other enterohemorrhagic 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 caytanensis*) were not recognized as causes of foodborne illness just 20 years ago, and some proportion of gastrointestinal illness is caused by foodborne agents that have not yet been identified. It is estimated that 62 million foodborne-related illnesses and 3,200 deaths occur in the United States each year from unknown pathogens.⁴⁴ *Bacillus anthracis*, although rarely seen as a gastrointestinal illness in the United States, has become a concern since cases occurred in 2000 (see

below). Even in areas of the world where gastrointestinal anthrax is more common, the oropharyngeal form is underreported because physicians are unfamiliar with it.⁴⁵ Unreported foodborne disease, deaths from unknown food agents,⁴⁶ and chronic sequelae⁴⁷ may be a huge unrecognized burden of illness.

Bacillus anthracis

B anthracis is the causative agent of anthrax, a naturally occurring zoonotic disease. The greatest bioweapon threat from anthrax is through aerosol dispersion and subsequent inhalation of concentrated spores (for more details see Chapter 4, 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 is rare in the United States and other developed nations.^{45,48} 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 persons (92%) developed presumed anthrax. Of these, 91% had gastrointestinal complaints and 9% had oropharyngeal edema; 9 of the victims, all children, died within 48 hours of illness onset.⁴⁸ Gastrointestinal anthrax can occur naturally in the United States, and anthrax-contaminated meat has been found to be associated with gastrointestinal illness in Minnesota as recently as 2000.⁴⁹ Purposeful contamination of food or water is possible but would require a high infective dose. Misdiagnosis may lead to a higher mortality in gastrointestinal anthrax than in other forms of the disease; thus, awareness of this disease remains important in anthrax-endemic areas and in the setting of possible bioterrorism.

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 1947.⁵⁰ *Campylobacter jejuni* (Figure 25-2), along with *C coli*, have been recognized as agents of gastrointestinal infection since the late 1970s. *C jejuni* is considered the most commonly reported foodborne bacterial pathogen in the United States, affecting 2.4 million persons annually.⁵¹ Campylobacteriosis is an enteric illness of variable severity, including symptoms of diarrhea (which may be bloody), abdominal pain, malaise, fever, nausea, and vomiting, occurring 2 to 5 days after exposure. Although many infections are

asymptomatic, infection with this pathogen has been associated with development of Guillain-Barré syndrome and arthritis.^{52,53} Infants are more susceptible to *C jejuni* infections upon first exposure.⁵⁴ Persons who recover from *C jejuni* infection develop immunity. Poultry colonized with *Campylobacter* species is a major source of infections for humans.⁵⁵⁻⁵⁸ The reported incidence of *Campylobacter* species on poultry carcasses has varied but has been as high as 100%.⁵⁷

Several virulence properties, including motility, adherence, invasion, and toxin production, have been recognized in *C jejuni*.⁵⁹ 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.⁶⁰ Although cytolethal distending toxin is the best-characterized *Campylobacter* toxin, its role in the pathogenesis of human campylobacteriosis is unclear.⁶¹

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, with 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 should receive antimicrobial treatment. Erythromycin, because it is safe, lacks serious toxicity, and is easy to administer, is the drug of choice for *C jejuni* infections. However, most clinical trials performed in adults or children

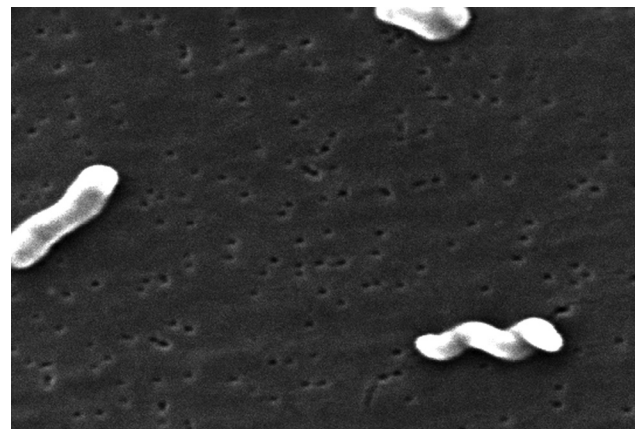


Fig. 25-2. Scanning electron microscope image of *Campylobacter jejuni* illustrating its corkscrew appearance. Photograph: Courtesy of Janice Carr, Centers for Disease Control and Prevention Public Health Image Library.

have not found that erythromycin significantly alters the clinical course of *Campylobacter* infections.^{62,63} 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.⁶⁴ 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.⁶⁵ In another study conducted in 1997 in Minnesota, 13 of 91 chicken products (14%) purchased in grocery stores were contaminated with ciprofloxacin-resistant *C jejuni*,⁶⁶ illustrating the need for more prudent antimicrobial use in food-animal production.

Clostridium botulinum

C 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.⁶⁷ There are seven antigenic types of toxin, designated A through G; most human disease is caused by types A, B, and E. Botulinum toxins A and B are most often associated with home canning and home-prepared foods, while botulinum toxin E is exclusively associated with ingestion of aquatic animals. 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.⁶⁸ From 1990 to 2000, 160 foodborne botulism events affected 263 persons in the United States. Of these patients, 67 required intubation, and 11 deaths occurred.⁶⁹ 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.⁶⁹

Escherichia coli O157:H7

E coli O157:H7 has emerged as a cause of serious pediatric illness worldwide. Its intrinsic Shiga toxins can initiate a cascade of events that include bloody

diarrhea and hemolytic uremic syndrome (HUS), exhibited by microangiopathic hemolytic anemia, acute renal failure, and thrombocytopenia.⁷⁰ HUS occurs in about 4% of all reported cases, and persons under five years of age are at greatest risk.⁴⁴ The mortality rate for HUS is 3% to 5%, and about 5% of survivors have severe consequences, including end-stage renal disease and permanent neurological damage.⁷¹ Antibiotic treatment of *E coli* O157:H7 is not recommended.⁷² There is anecdotal evidence for an increase in the risk of HUS with the use of some antimicrobial agents, although conclusive proof of this occurrence is lacking. Fluid replacement is the cornerstone of the treatment of diarrheal illness caused by the enterohemorrhagic *E coli*.

The primary source of *E coli* O157:H7 is beef cattle. The current animal culture practice of feeding grain (rather than hay) to these animals decreases the pH in their colons, thereby promoting acid-resistance and enhanced growth of *E coli* pathogens.⁷³

Salmonella Species

Salmonella species infect an estimated 1.4 million persons annually in the United States. Although most infections are self-limiting, with diarrhea, vomiting, abdominal cramps, and fever, severe infections are not uncommon. Estimates suggest that approximately 15,000 people are hospitalized and over 500 deaths occur each year from *Salmonella* infections.⁴⁴ Food animals are the primary reservoir for human nontyphoidal *Salmonella* infections. There are thousands of *Salmonella* serotypes, and many naturally inhabit avian, mammalian, and reptilian gastrointestinal tracts. Poultry is the main source of the salmonellae in the food supply; other vehicles for disease transmission include raw salads, milk, water, and shellfish.

Infection with many *Salmonella* serotypes causes gastroenteritis with associated diarrhea, vomiting, febrile illness, headache, and dehydration. Septicemia, enteric fever, and localized infections may also evolve from salmonellosis infection. The most highly pathogenic of the salmonellae, *S typhi*, causes typhoid fever, which includes symptoms of septicemia, high fever, headache, and gastrointestinal illness. *S typhimurium* was the pathogen used in 1984 by an Oregon cult to cause illness by purposeful contamination of salad bars.⁷⁴ Over 750 cases of illness resulted, but no deaths occurred, which may not have been the case if *S typhi* had been used. 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 food distribution system.⁷⁵

Tick-borne Diseases

Borreliosis

Lyme arthritis, as a distinct clinical entity, was recognized as early as 1972 in residents of three communities in eastern Connecticut.⁷⁶ 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 first observed spirochetes in adult *Ixodes scapularis* (then called *I dammini*) ticks collected from vegetation on Shelter Island, New York, a known endemic focus of Lyme disease.⁷⁷ The bacteria were shown to react specifically with antibodies from Lyme disease patients,⁷⁷⁻⁷⁹ and later, spirochetes were isolated from the blood of two patients with Lyme disease,⁸⁰ proving the spirochetal etiology of the infection.⁷⁸ The spirochetes were later named *Borrelia burgdorferi* (Figure 25-3), after the discoverer. The deer tick, *I scapularis*, is now known to be the primary vector of Lyme disease in the northeastern and north central United States (Figure 25-4). Other vectors are closely related ixodid ticks; 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.⁸¹ 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

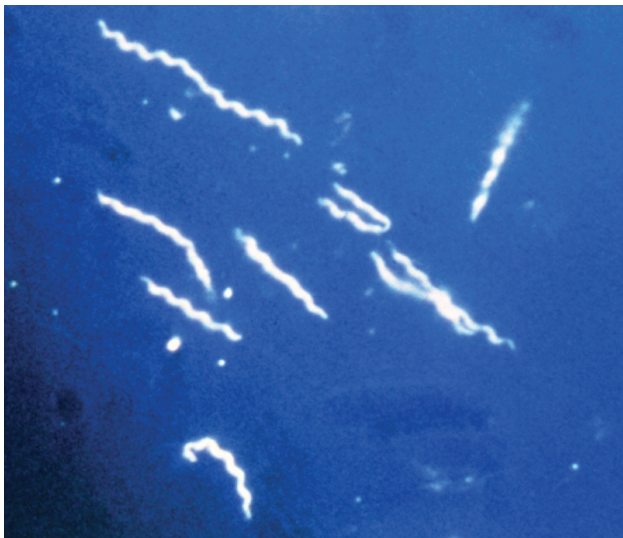


Fig. 25-3. Darkfield photomicrograph of the Lyme disease spirochete, *Borrelia burgdorferi*, magnified 400x. Photograph: Courtesy of Centers for Disease Control and Prevention Public Health Image Library.



Fig. 25-4. *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. Image 1669. Reproduced from: Centers for Disease Control and Prevention Public Health Image Library Web site. Photograph by Jim Gathany and provided by Michael L Levin, PhD. Available at: <http://phil.CDC.gov>. Accessed April 6, 2007.

most European disease results from the latter two. In Asia, only *B afzelii* and *B garinii* seem to be associated with the illness.^{81,82} *B japonica*, which was isolated in Japan, is not known to cause human disease.⁸³

Lyme disease evolves from a red macule or papule that expands annularly like a bulls-eye rash, defined as erythema migrans, which may exhibit as a single lesion or as multiple lesions. 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 onset of erythema migrans, 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 may result.⁷²

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 2001.⁸⁴ In 2002, 23,763 cases were reported, an increase of 40% from the previous year.⁸⁴ As with other tick-borne diseases, this continuing 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) very similar to, and often indistinguishable from, that seen in Lyme disease has been identified in the southeastern and south central United States.⁸⁵⁻⁸⁷ Unlike Lyme disease, however, symptoms develop following the bite of the lone star tick, *Amblyomma americanum* (Figure 25-5). The disease is named southern tick-associated rash illness (STARI), but has also been referred to as Master's disease, or southern Lyme disease. *Ambly-*



Fig. 25-5. 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. 2003. Image 4407.

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omma americanum ticks are not known to be competent vectors of *B burgdorferi*, and serologic testing for Lyme disease in STARI patients is typically negative, despite microscopic evidence of spirochetes in biopsy samples. This finding led to speculation among physicians and researchers 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 STARI.⁸⁸⁻⁹¹ The organism, named *Borrelia lonestari*, was initially described only by polymerase chain reaction amplification of the flagellin B gene and 16S ribosomal DNA,⁹² but has now been isolated in culture and more extensively studied.⁹³

Still other species of *Borrelia* known to cause relapsing fever 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*, the causative agent of tick-borne relapsing fever, is transmitted by the soft tick *Ornithodoros hermsii*.⁹⁴ The disease results in fever lasting 2 to 9 days with 1 to 10 relapses. Although the total duration of louse-borne disease usually averages 13 to 16 days, the tick-borne disease often lasts longer. Gastrointestinal and respiratory involvement is common. Neuropsychiatric symptoms also have been known to occur.⁷² Relapsing fever, first reported in the United States in 1915,⁹⁵ normally occurs in the higher elevations of the western United States and southern British Columbia, Canada. After a relapsing fever outbreak among five persons visiting a cabin in western Montana,⁹⁴ spirochetes isolated from two of the patients were identified as *B hermsii*, and *O hermsii* ticks were collected from the cabin in which the patients had slept. This was the first report of both *B hermsii* and *O hermsii* in Montana, suggesting the risk of infection may be expanding beyond the previously recognized geographic range.

Ehrlichiosis

Human granulocytic ehrlichiosis 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, and granulocytic ehrlichiosis occurs in areas where Lyme disease is endemic.⁷² The *Amblyomma americanum* tick (see Figure 25-5) transmits *E chaffeensis*, and *I scapularis* (see Figure 25-4), the Lyme disease vector, also transmits *A phagocytophilum*. A spectrum of mild to severe, life-threatening, or fatal disease (< 1% mortality) occurs with ehrlichiosis. 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.⁷²

Because the *Ixodes scapularis* tick is the vector for transmission of *B burgdorferi*, *A phagocytophilum*, and *B microti*, coinfections of Lyme disease, ehrlichiosis, and babesiosis (caused by the protozoan *Babesia microtii*) can be transmitted by a bite from this tick. Ticks of the *Ixodes* genus can transmit all of these diseases as well as tick-borne encephalitis.⁷² Coinfections with babesiosis and Lyme disease are known to sometimes increase the severity of both diseases.⁷²

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.⁹⁶ However, the number of resistant organisms, the geographic regions affected by drug resistance, and the number of bacterial species that are multidrug resistant is increasing. Since the 1980s, a reemergence of tuberculosis has occurred that often results from drug-resistant *Mycobacterium tuberculosis*⁹⁷ and requires the use of several (sometimes six to seven) different drugs to treat.⁹⁸ Other notable examples of multidrug resistant strains worldwide include *Enterococcus faecium*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *S aureus*, *Acinetobacter baumannii*, and *P aeruginosa*.⁹⁶ In developing countries, multidrug resistant enteric bacteria such as *Salmonella enteritidis*, *Shigella flexneri*, and *V cholerae*

are major threats to public health.

Salmonella antibiotic resistance has emerged as a serious concern in agriculture as well as patient management.⁹⁹⁻¹⁰¹ 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 some biocides.¹⁰²

Few antibiotics are more potent than vancomycin. The emergence of microbial vancomycin resistance has been of increasing concern to clinicians and public health professionals during the past decade, and surveillance systems have been instituted to monitor these pathogens.¹⁰³ *Staphylococcus aureus* is an important cause of illness and death, accounting for about one fifth of bacteremia cases in the United States.¹⁰⁴ The discovery of vancomycin resistance in *S aureus* clinical isolates in the United States could portend the end of the antibiotic era in medicine.^{105,106}

Both hospital and home healthcare patients are significantly affected by the growing emergence of antibiotic resistance.^{107,108} 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.¹⁰⁹ Vancomycin-intermediate resistance among *S aureus* has also been identified, and subsequent guidance has been developed for its identification and control of transmission.¹¹⁰ Appropriate antibiotic use will continue to be an important issue for clinicians and epidemiologists for the foreseeable future.¹¹¹

EMERGING VIRAL DISEASES

Avian Influenza and the Threat of Pandemic Influenza

Influenza is a highly contagious, acute respiratory illness caused by one of the oldest viruses known, with clear evidence of disease dating back to the Middle Ages and probably occurring as early as ancient Greece and Rome. The virus, a member of the *Orthomyxoviridae* family, contains a segmented negative-sense RNA genome, with each segment corresponding to a gene. The segmented nature of the genome allows for the reassortment or exchange of segments (and genes) between two virus strains coinfecting the same cell. Thus, by their very nature, influenza viruses are constantly reemerging through changes in their genetic make-up. Influenza virus strains that cause pandemics are classical examples of emerging viruses. There are three main types of influenza viruses, termed influenza A, B, and C; however, only influenza A

has been associated with human influenza pandemics. Two genes of special importance encode for the surface proteins hemagglutinin (HA) and neuraminidase (NA). These proteins, seen as spikes in electron micrographs (Figure 25-6), are major antigens of the virus and are involved with the interactions between the virus and host cells. Because of their importance, subtypes of influenza A viruses are often designated by their particular HA and NA types (to date, distinct hemagglutinin subtypes of influenza B and C viruses have not been observed). There are 15 HA and 9 NA subtypes, with each subtype differing by 30% or more in amino acid sequence homology.¹¹² All of these subtypes are found in wild waterfowl, which act as the reservoir host for influenza A viruses. Thus far, only viruses carrying one of three HA subtypes (H1, H2, H3) have crossed species barriers and established themselves in humans (H7 and H9 subtype viruses

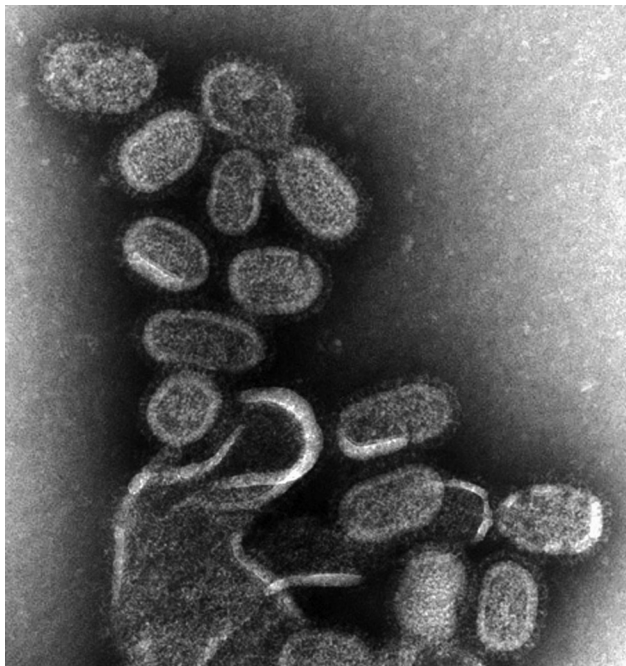


Fig. 25-6. Negative-stained transmission electron micrograph showing the reconstructed 1918 influenza virions that were collected from the supernatants of virus-infected Madin-Darby canine kidney cell culture 18-hours postinfection. Surface spikes (hemagglutinin and neuraminidase) can be clearly seen extending from the surface of the virions. 2005. Image 8160.

Reproduced from: Centers for Disease Control and Prevention Public Health Image Library Web site. Photograph by Cynthia Goldsmith and provided by Dr Terrence Tumpey. Available at: <http://phil.CDC.gov>. Accessed April 6, 2007.

have caused human infections, although rarely). For example, one circulating influenza strain, designated subtype H3N2, has been the most commonly isolated strain during the past 36 years.

Variants of influenza A viruses can result from mutation 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, which occur randomly as the virus is copied in infected cells, are largely responsible for the annual epidemics of influenza seen during the winter months. Another type of viral change is antigenic shift, which results from the reassortment of genes that occurs when two different influenza viruses infect the same host cell. This phenomenon results in the emergence of new pandemic influenza A strains. Since 1933, when the virus was first isolated (an H1N1 subtype), major antigenic shifts (and pandemics) have occurred in 1957 (“Asian influenza,” an H2N2 subtype) and in 1968

(“Hong Kong influenza,” an H3N2 subtype). After a hiatus of more than 20 years, the H1N1 subtype virus reappeared in 1977. That year it did not result in severe disease, however, most likely because of the immunity of persons over 20 years of age who had been infected with the virus earlier in the century. It is highly unlikely that this virus was maintained in an animal host for over 20 years without changes; possibly, the virus was maintained in a freezer until it was somehow reintroduced into the human population. Retrospective seroepidemiological analysis can provide indications of the virus subtypes circulating during epidemics and pandemics that occurred before 1933. For instance, the 1889–1890 influenza epidemic was caused by a virus antigenically similar to the 1957 Asian strains (H2N2).¹¹³ Likewise, the epidemic of 1900 may have been caused by a virus with an HA similar to the H3N2 pandemic virus of 1968.

Of the three influenza pandemics that occurred in the 20th century, the pandemic of 1918–1919 was the most devastating, causing an estimated 20 million to 40 million deaths worldwide. Unusually, healthy young 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. A reemergent 1918-like influenza virus would have even more devastating effects in today’s era of rapid jet transportation and overpopulation. The pandemic killed an estimated 675,000 Americans, including 43,000 servicemen mobilized for World War I (Figures 25-7 and 25-8), and may have played a role in ending the war.¹¹⁴ Its impact



Fig. 25-7. 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.

was so profound that the average US life expectancy temporarily declined by over 10 years.¹¹⁵

Analysis of survivor antibody titers from the late 1930s suggested that the 1918 strain was an H1N1 subtype closely related to classic swine influenza virus.¹¹⁶ This identification was confirmed by researchers at the Armed Forces Institute of Pathology in Washington, DC, who analyzed influenza viral RNA obtained from preserved lung tissue of US servicemen who died during the 1918 pandemic.¹¹⁷ Since the original work on the HA gene, several other 1918 influenza virus genes have been sequenced and characterized.¹¹⁸⁻¹²¹ Unfortunately, no obvious genetic changes were observed in any of these gene sequences that would account for the exceptional virulence of the pandemic virus.^{122,123} However, the recent solving of the crystal structure of the HA protein derived from reassembly of extinct 1918 influenza virus may help explain the mystery.^{124,125} For instance, although the 1918 virus' HA protein is

distinctly avian in structure, particularly within the receptor binding site, it is able to form structural conformations that bind to human cells. This may explain how the virus could have been so virulent (because of the avian-like structure of its HA protein) and, at the same time, spread through the human population with such ease. In addition, in 2005, a team of researchers succeeded in reconstructing the 1918 pandemic virus by using gene sequences obtained from a 1918 victim (see Figure 25-6). The reconstructed virus was highly virulent, killing mice more quickly than any other human influenza virus known.¹²⁶ Such research efforts may shed more light on the highly virulent nature of the 1918 virus and help in the development of vaccines and treatments for future pandemic influenza viruses.

Wild aquatic birds, the reservoirs of all subtypes of influenza A virus, are generally unharmed by the virus. It had been thought that these purely avian influenza viruses, although highly pathogenic for domestic poultry, did not replicate efficiently or cause disease in humans. Before the late 1990s, there were only three reported isolations of avian influenza viruses from humans. The first was from a patient with hepatitis in 1959.¹²⁷ The other two were cases of conjunctivitis, one of which was in a laboratory worker in Australia who developed infection after accidental exposure directly in the eye,¹²⁸ and the second in an animal handler who had direct contact with an infected seal.¹²⁹ All of these cases were associated with H7N7 subtype viruses. In contrast to the rarity of H7N7 avian viral isolations from humans, serosurveys of farmers in rural southern China suggest that many other subtypes of avian viruses have crossed the species barrier and infected humans.¹³⁰ Specifically, seroprevalence levels of 2% to 7% for H5 viruses alone have been reported,¹³⁰ and the seropositivity of human sera for H7, H10, and H11 viruses was estimated to be as high as 38%, 17%, and 15%, respectively.¹³⁰ It has long been believed that avian viruses could not efficiently infect humans because of receptor specificity, preventing the emergence of new pandemic strains via direct avian-to-human transmission. Transmission from aquatic birds to humans was hypothesized to require infection of an intermediate host, such as a pig, that has both human-specific and avian-specific receptors on its respiratory epithelium. Pigs were considered "mixing vessels," allowing for the reassortment between avian and human influenza viruses to occur.

However, human cases of avian influenza have recently become increasingly frequent. In 1996 an H7N7 virus was isolated from a woman who kept ducks and had conjunctivitis in her eye.¹³¹ The source

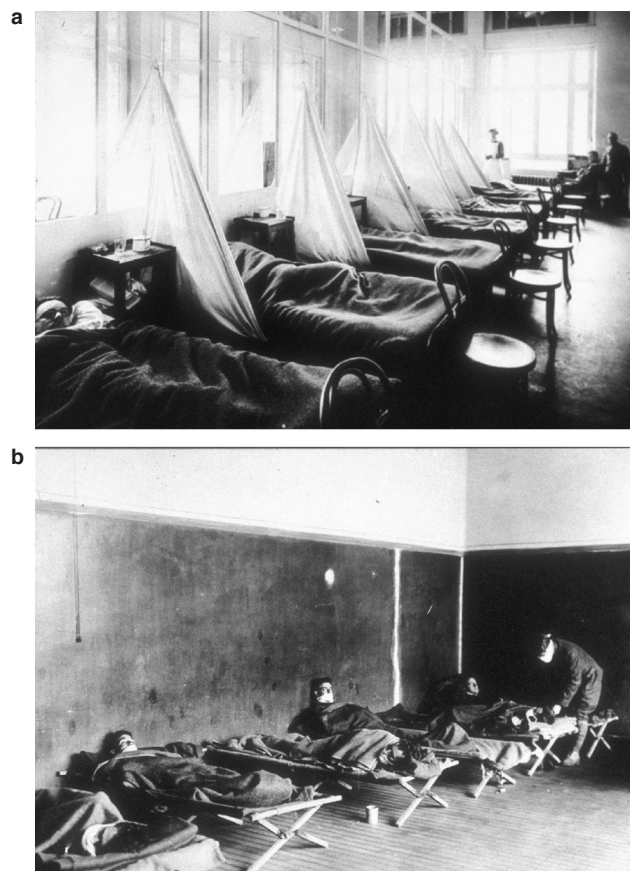


Fig. 25-8. Influenza wards, US Army camp hospitals at (a) Aix-Les-Bains, France (Reeve 14682), and (b) Hollerich, Luxembourg (Reeve 15183).

Photographs: Courtesy of the Otis Historical Archives, National Museum of Health and Medicine, Washington, DC.

of the virus was considered to be waterfowl because she tended a collection of 26 ducks of various breeds that mixed freely with wild waterfowl on a small lake. In the spring of 1997, an H5N1 virus was isolated from a 3-year-old boy who died in Hong Kong.¹³² By the end of the same year, a total of 18 people were infected with the same H5N1 virus, and six died. Genetic analysis of these viruses showed that all of the viral genes were of avian origin (ie, they were not reassortants), and epidemiological evidence strongly suggested that direct contact with infected poultry was the route of transmission.^{133,134} In addition, they appeared to be identical to viruses first isolated from an outbreak in chickens in Hong Kong earlier that same year. Because human populations lacked immunity to the H5 influenza virus subtype, there was great concern about the possibility of a major pandemic from this newly emergent virus. Fortunately, however, prompt and thorough culling of poultry on affected farms throughout Hong Kong stopped the outbreak in poultry, and enforcement of personal protection procedures for poultry handlers stopped the transmission of the novel virus to humans. In addition, the lack of evidence for human-to-human transmission in the majority of cases in Hong Kong suggested that the virus had not fully adapted to its human host.

In 2003 an H5N1 virus was isolated again in Hong Kong from a father and son who presented with respiratory illness after returning from mainland China.¹³⁵ A daughter and the mother of this family also became ill, and the daughter died while visiting mainland China. The father ultimately died of viral pneumonia, although the boy eventually recovered. Meanwhile, in Europe, outbreaks of highly pathogenic H7N7 viruses on poultry farms in the Netherlands resulted in the culling of over 30 million chickens before the virus was contained.¹³⁶ In addition, some 450 people had reported health complaints, including conjunctivitis and influenza-like illness, and a veterinarian who visited one of the farms developed high fever and severe headache, and died of respiratory distress syndrome 15 days later.¹³⁶

Since late 2003 outbreaks of an Asian strain of highly pathogenic avian influenza (H5N1) have caused lethal illness among poultry throughout southeast and central Asia.¹³⁷ Most of these countries were experiencing highly pathogenic avian influenza for the first time. By the end of 2005, the outbreak resulted in 132 reported human cases, 68 of which were fatal.¹³⁸ In 2005 the range of the virus extended out of Asia and into Europe, with several human infections in Turkey, causing concern that a new virus subtype with pandemic potential could emerge.

Severe Acute Respiratory Syndrome

SARS is a new infectious disease that first emerged in Guangdong province of China in November 2002. Initially referred to as “infectious atypical pneumonia” by Chinese clinicians, SARS was later provided a case definition and its current name by the World Health Organization. The disease usually began with high fever and mild respiratory distress, but rapidly progressed to pneumonia within a few days. 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, where he transmitted the infection to 16 other guests. These individuals quickly spread the disease in Hong Kong, Singapore, Vietnam, and Toronto.¹³⁹ Within weeks, SARS had spread to affect thousands of people in 25 countries across five continents. By the end of the global outbreak in July 2003, there were over 8,000 recorded cases, with 744 fatalities.¹⁴⁰ By the end of March 2003, a novel coronavirus (SARS-CoV) was identified as the infectious agent of the syndrome.¹⁴¹⁻¹⁴³ Although researchers in China observed coronavirus-like particles in cultures grown from patient samples from Guangdong in mid-February, Chinese officials at the time reported that a *Chlamydia* bacterium caused the disease, and the coronavirus results were not reported.¹⁴⁴

Where did the SARS-CoV originate and how did it become a highly lethal human pathogen? The exact origin of the SARS-CoV is still a mystery; however, the disease probably first emerged in Guangdong around November 2002.^{145,146} One of the first identified SARS patients was a chef from Heyuan who worked at a restaurant in Shenzhen. As a chef, he came into regular contact with several types of live animals used as exotic game food. This prompted speculation that SARS might be a zoonotic disease. Guangdong province is famous for its “wet markets,” where a wide variety of vertebrate and invertebrate animals are housed together and sold for their medicinal properties or culinary potential.⁷ More than one third of the early SARS cases were among food handlers.¹⁴⁷ Studies with avian influenza viruses in live poultry markets have shown that such viruses amplify within the setting of a market trading in live birds.¹⁴⁸ Lack of serologic evidence of previous infection in healthy humans suggested that SARS-CoV had recently emerged in the human population and that animal-to-human interspecies transmission might be a reasonable explanation for its emergence. Further support for a zoonotic origin of SARS came from the initial isolation of a SARS-like coronavirus from Himalayan palm civets (Figure 25-9) found in a live animal market



Fig. 25-9. 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.

in Guangdong, China.¹⁴⁹ However, subsequent surveys failed to find the virus in either farmed or wild civets, suggesting the civet may have served only as an amplification host for the virus. In 2005 two research teams independently identified the Chinese horseshoe bat (*Rhinolophus sinicus*) as the natural viral reservoir from which the SARS coronavirus that infected humans likely emerged.^{150,151} Many people in Asia eat bats or use their feces for medicinal purposes. The researchers speculate that bats may have first passed the viruses to animals in the wild or in the live animal markets of southern China where bats are sold as food.

Emerging Paramyxoviruses

Hendra Virus

In 1994 a new member of the paramyxoviruses emerged in Brisbane, Australia, killing 14 race horses and a horse trainer.^{152,153} 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.¹⁵⁴ 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. The virus was therefore initially named equine morbillivirus,¹⁵⁵ but was later renamed Hendra virus after the Brisbane suburb where the outbreak occurred. Serologic¹⁵⁶ and other evidence of infection was found in several species of Australian flying foxes (ie, fruit bats of the genus *Pteropus*) (Figure 25-10), 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 coevolved with its pteropid hosts.¹⁵⁷⁻¹⁵⁹

Additional occurrences of Hendra virus have been rare, sporadic, and limited to horses. In 1999 a horse from near Cairns in northern Queensland died from Hendra disease,^{160,161} and in 2004 Hendra virus was confirmed in another dead horse from Townsville, also in northern Queensland.

Nipah Virus

Nearly 5 years after the discovery of the Hendra virus, a massive outbreak of porcine respiratory disease in Malaysia caused the deaths of 105 pig farm or abattoir workers and the eventual culling of over 1 million pigs, leading to the discovery of a new virus closely related to Hendra, called Nipah virus.¹⁶² The predominant clinical syndrome in humans was encephalitic (unlike the respiratory syndrome seen in the infected pigs), with clinical signs including fever, headache, myalgia, drowsiness, and disorientation, sometimes leading to coma within 48 hours.^{163,164} The majority of human cases included a history of direct contact with infected pigs; most were among pig farmers. Preliminary research on the new virus revealed ultrastructural, antigenic, serologic, and molecular characteristics similar to Hendra virus.¹⁶² Follow-up molecular studies showed the genome of Nipah virus to be highly homologous to that of Hendra virus, with specific genes having nucleotide homologies between 70% and 88%, and amino acid homologies ranging from 67% to 92%.¹⁶⁵ Given the degree of similarity and other unique features of these viruses, both were placed in a new genus, *Henipavirus*, within the family *Paramyxoviridae*.¹⁶⁶ Because of the similarities between Nipah and Hendra viruses, attention focused on Malaysian bats as the source of the infection in pigs.¹⁵⁷ 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).¹⁶⁷ 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



Fig. 25-10. 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.

colony of seropositive flying foxes from Tioman Island off the coast of Malaysia.¹⁶⁸

The virus reemerged in Bangladesh in two separate outbreaks in 2001 and 2003, each resulting in a cluster of febrile neurological illnesses with nine and eight reported deaths, respectively.¹⁶⁹ In contrast to the outbreaks in Malaysia, where animal illnesses were reported and close contact with pigs was strongly associated with Nipah virus infection, no obvious zoonotic source was identified in Bangladesh. However, antibodies to Nipah virus were detected in two local *Pteropus* bats, so inadvertent direct contact with bats or bat secretions is a possible explanation for the infection (see Figure 25-10).

Menangle and Tioman Viruses

Menangle virus is a rare, previously undescribed virus that caused a single episode of reproductive disease in pigs in a large commercial piggery near Sydney, Australia, in 1997.¹⁷⁰ The virus caused stillbirths with deformities and occasional abortions in the affected pigs. Affected stillborn piglets frequently had severe degeneration of the brain and spinal cord. No disease was observed in postnatal animals of any age, although over 90% of them had high titers of neutralizing antibodies to the virus. Two persons who worked with

the pigs developed influenza-like illness with sudden onset of malaise, chills, fever, severe headaches, and myalgia.¹⁷¹ Convalescent-phase serum samples from both patients were found to have high titers of neutralizing antibodies to the virus, and extensive serologic testing showed no evidence of any alternative cause for their symptoms. Again, fruit bats were identified as the probable source of infection.¹⁷⁰ A large breeding colony of gray-headed and red fruit bats was found roosting within 200 meters of the affected piggery, and serum samples collected from these bats were positive for neutralizing antibodies against Menangle virus.¹⁷⁰

During the search for the natural host of Nipah virus, another new member of the Paramyxoviridae family, Tioman virus, was isolated from the urine of flying foxes found on Tioman Island.¹⁷² Nucleotide sequence and phylogenetic analysis indicate that Tioman and Menangle viruses are closely related; however, the potential of Tioman virus to cause disease in animal and humans is unknown.

Emerging Arthropod-borne Viruses: Dengue and West Nile Viruses

Mosquito-borne viruses are members of the more general category of arthropod-borne 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. Still other infections are known to cause rash and/or 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.¹⁷³ Because of 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 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 stay within their previously known geographical regions.

Dengue Virus

Dengue is caused by one of four viral subtypes (designated DENV-1 to DENV-4) and is one of the most common mosquito-borne 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.¹⁷⁴ Infection with dengue virus can present in several clinical manifestations. Classical dengue fever is an acute febrile illness that often occurs in children, characterized by fever, severe headache and muscle aches, nausea, vomiting, and rash. This acute illness usually lasts for 8 to 10 days and 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.¹⁷⁵ DSS occurs if the patient goes on to develop hypotension and shock from plasma leakage and circulatory failure. This happens in about one third of severe dengue cases (especially in 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.¹⁷⁵ 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, known as immune enhancement,^{176,177} suggests that patients experiencing a second infection with a heterologous DENV serotype have a significantly higher risk of developing DHF/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.¹⁷⁵ The other theory assumes that dengue viruses change genetically as a result of selective pressures as they replicate in humans and/or mosquitoes and that the phenotypic expression of these genetic changes may include increased virus replication and virulence. All the data taken together suggest that a combination of age and the viral, immunopathogenic, and genetic background of the person play a role in disease severity.¹⁷⁴

Although dengue viruses were first identified in southeast Asia in the 1940s and 1950s, evidence suggests that they derived from a primitive progenitor introduced to Asia from Africa about 1,000 years ago.¹⁷⁸ Studies of dengue virus ecology in sylvatic habitats of western Africa and Malaysia have identified transmission cycles involving nonhuman primates as reservoir hosts and arboreal, tree-hole dwelling *Aedes* species mosquitoes as vectors.^{179,180} Efficient interhuman dengue virus 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 that the sylvatic cycle is probably ancestral.¹⁸¹ Further support for this idea comes from studies suggesting that a zoonotic transfer of dengue virus from sylvatic to sustained human transmission occurred between 125 and 320 years ago.¹⁷⁸ In the past 300 years, these viruses have become established in the urban centers of the tropics. The principal urban vector, *A. aegypti*, is highly domesticated and adapted to humans, preferring to feed on people and lay their eggs in artificial containers in and around houses. *A. albopictus* (the Asian tiger mosquito [Figure 28-11]) is a secondary vector of dengue viruses. Dengue occurs rarely in the United States, primarily in southern Texas. However, because the vectors are distributed throughout much of the southeastern United States, a greater potential for future emergence of dengue in the United States exists.

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.^{175,182,183} The reasons for this are not fully understood, but are thought to stem from major demographic and societal changes over the past 50 years, particularly the unprecedented global population growth and associated unplanned and uncontrolled urbanization, especially in the tropical developing countries.¹⁷⁵ Other potential factors associated with the global emergence of dengue include the lack of



Fig. 25-11. A female *Aedes albopictus* mosquito feeding on a human host. These mosquitos, along with *A aegypti*, are competent vectors of dengue virus. 2003. Image 4490. Reproduced from: Centers for Disease Control and Prevention Public Health Image Library Web site. Photograph by James Gathany. Available at: <http://phil.CDC.gov>. Accessed April 6, 2007.

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.¹⁷⁵

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 mosquito-borne arboviruses, found in areas throughout Africa, Europe, Asia, and North America (Figure 25-12). Yet until recently, it was completely exotic to the western hemisphere. In 1999 WNV emerged in the New York, New York, area as the cause of an outbreak of meningoencephalitis resulting in 7 deaths among 62 confirmed cases.¹⁸⁴ There was a concurrent outbreak among the horse population on Long Island, New York, resulting in 25 equine cases including 9 fatalities.¹⁸⁵ The principal mosquito vectors were likely *Culex pipiens* or other related *Culex* species; however, the virus has been isolated from a number of other mosquito species and even, in some cases, from ticks.^{186,187} The virus has been shown to be capable of infecting over 50 species of mosquitoes and ticks.^{187,188} Since the introduction of WNV into New York in 1999, the virus has spread across the United States (Figure 25-13). In addition, since 2000, WNV has spread into Central America, with virus being isolated in Mexico, El Salvador, and

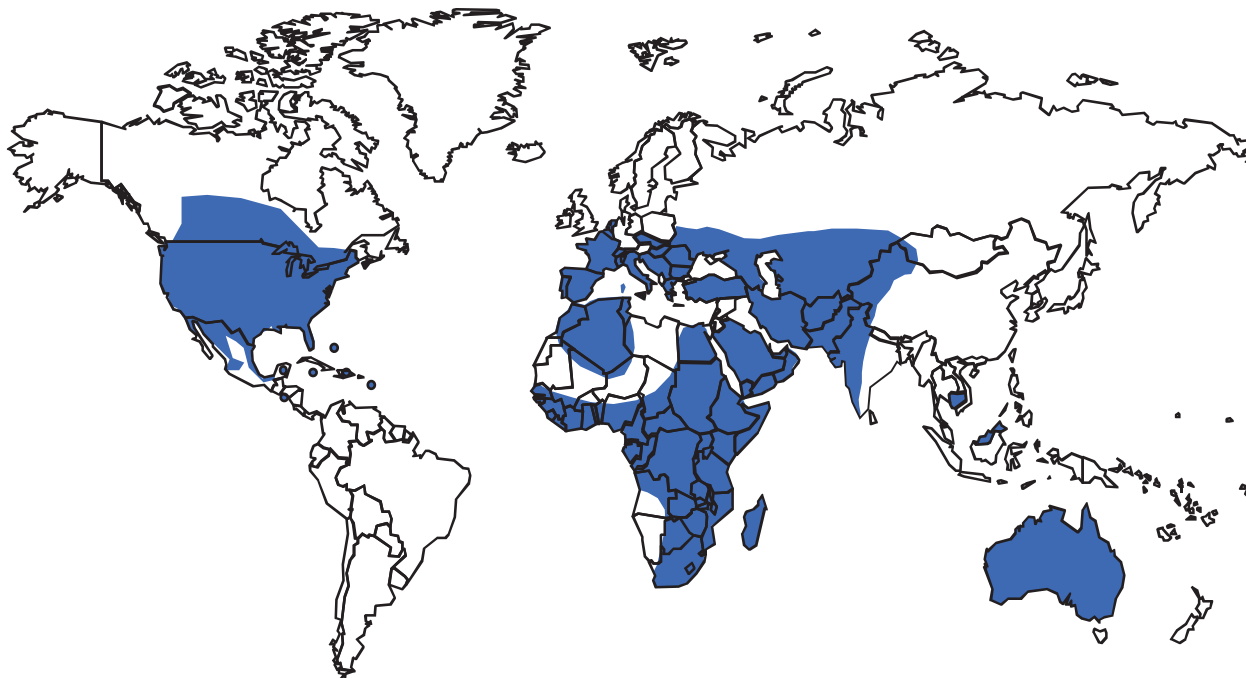


Fig. 25-12. Approximate geographic range of West Nile virus, 2004. Map: Courtesy of Dr Robert Lanciotti, Arbovirus Diseases Branch, Centers for Disease Control and Prevention, Fort Collins, Colorado.

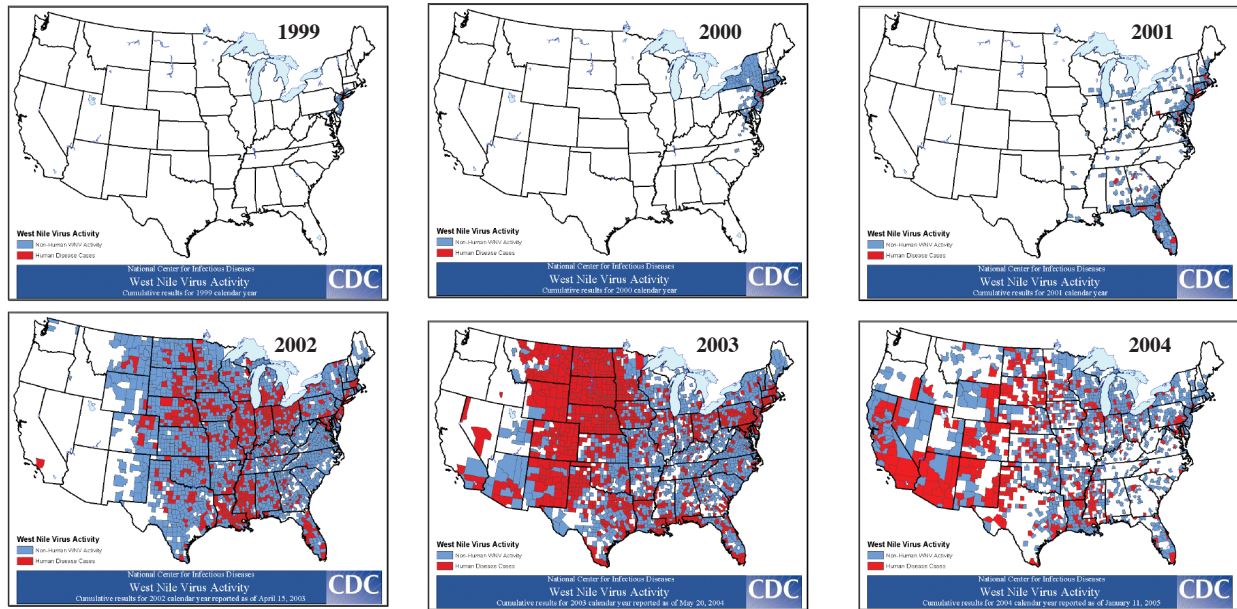


Fig. 25-13. Spread of West Nile virus activity across the United States, 1999 to 2004. Data represent nonhuman West Nile virus activity (in blue) and human disease cases (in red) in the United States by county. Reproduced from: National Center for Infectious Diseases, Centers for Disease Control and Prevention.

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 by organ transplantation, causing even greater public health concerns.¹⁸⁹⁻¹⁹⁴

GENETICALLY ENGINEERED THREATS

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 role has 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 to change during the course of disease outbreaks. In Europe, several generations of exposure to smallpox and measles ensured the survival of those most resistant to these diseases; when the diseases were introduced in the New World, unchecked contagion and decimation of the unexposed populations occurred.^{195,196} A classical example of agent-host adaptation in animals was the intentional introduction of myxomatosis (an orthopoxvirus similar to smallpox that infects rabbits) into Australia in an attempt to control or eliminate a scourge of rabbits. At first, rabbit mortality was very

high, but in time the rabbits acquired a degree of genetic resistance. In parallel, virulence diminished in the circulating virus, which persisted and was shed over a longer period of time in infected rabbits.¹⁹⁷ For both rabbit and virus, natural selection favored survival of the species. Humans have intentionally disturbed this “natural order,” from using relatively benign forms of disease as vaccines against the most virulent forms (eg, variolation, or the classical adaptation of measles, mumps, and rubella vaccines) to selecting the most virulent disease agents for biological weapons programs (the latter was finally stigmatized and outlawed 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.¹⁹⁸

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 reshuffling of genes between and among microbial agents and higher

organisms, has proven like so many technologies to have capacity for both good and ill. A few examples from the scientific literature illustrate the seriousness of the threat of genetically engineered microorganisms.

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. 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.¹⁹⁹⁻²⁰¹ Unauthorized conduct of most such experimentation has become not only difficult but illegal, subject to fines and incarceration, in many countries including the United States.

However, skilled laboratory researchers can now easily manipulate viral genomes by recovering infectious viruses from DNA clones. The progression of this technology with human pathogens began about 20 years ago with the simpler viruses (positive-sense, single-strand viruses with small genomes), such as poliovirus,²⁰² alphaviruses,²⁰³ and flaviviruses.²⁰⁴ The technology has grown to include negative-strand viruses (eg, vesicular stomatitis virus, respiratory syncytial virus, Ebola virus, and Crimean-Congo hemorrhagic fever virus) and segmented viruses (eg, influenza virus). Even the relatively huge genome of vaccinia virus has yielded to artificial resuscitation from DNA cloned into bacteria.²⁰⁵ In an experiment that was alarming to some observers in its simplicity, the capacity to derive a human pathogenic virus (poliovirus) by chemical synthesis was demonstrated.²⁰⁶ Even more controversial are the efforts to genetically resurrect the deadly 1918 influenza virus²⁰⁷⁻²¹⁰ and the proposals to genetically manipulate smallpox virus.²¹¹

In addition to the potential for recovering hazardous viruses from DNA clones, risks of accidental or malevolent outcomes are further elevated with engineered recombinant viruses. Experiments designed to create or improve vaccines, to understand interactions between virus and host, or to unveil some mysteries of the viruses themselves have simultaneously proven the ease with which bioactive and sometimes harmful molecules may be inserted into viruses. A large body of work with recombinant poxviruses was considered benign until a mouse poxvirus (ectromelia virus) rendered more virulent by its modification to coexpress a molecule of the immune system (interleukin-4) was reported.²¹² This result was merely part of a progres-

sion of studies of similar design and outcome,²¹³ but its timing (2001) crystallized the potential problem. This technology, applied to a wide array of human pathogens, remained underappreciated until federal regulators began defining and implementing safety and biosurety rules for select agents.

Ultimately, the capacity to create deadly and possibly even apocalyptic new organisms through genetic engineering is restrained largely by technical knowledge and opportunity, and also by awareness and intent. That is, techniques easily accomplished by skilled scientists are extremely difficult for the untrained and unequipped. However, a determined person with the appropriate knowledge and skills may succeed in malevolent creation of 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? New laws and regulations to emphatically restrict accidental or intentional creation of new deadly organisms, or possession of the deadly agents existing in nature, have already been imposed in the United States (eg, Public Law 107-188²¹⁴), 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 deadly pandemic influenza virus; in the case of the outbreak of a contagious genetically engineered microorganism, classical methods of epidemiology and quarantine would likely be helpful. Also encouraging is the application of the newest technologies to both diagnostics and bioforensics, likely shortening the time in which the nature and design of a newly emerged causative agent would remain unknown. Unfortunately, development of specific medical countermeasures (vaccines, therapeutic drugs) for a previously unknown organism can take months and usually years. One response to this problem is to fund the search for generic methods of boosting innate immunity 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. Even if medical countermeasures were nominally available, however, both genetically engineered and conventional agents could cause great localized harm and widespread panic.

SUMMARY

Emerging infectious diseases are among the most important future threats facing both military and civilian populations. These are diseases caused by

a variety of infectious agents (ie, bacteria, viruses, fungi, and parasites), some completely new to mankind, and others only newly recognized. Still others

may be common commensals that have acquired virulence factors (eg, toxins) or antimicrobial resistance genes through natural or unnatural (ie, genetic engineering) means.

Despite many successes in disease control and prevention in the 20th century, infectious diseases remain the leading cause of death worldwide and the third leading cause of death in the United States. AIDS, which was first recognized in 1981, is the most dramatic example of a new infectious disease that has emerged rapidly in the past 25 years. The AIDS pandemic will continue to put large numbers of people at risk for new and reemerging opportunistic infections. The rapid spread of the WNV across the United States after its introduction in 1999 and the increasing problem of antimicrobial resistance are other examples of microbes' ability to emerge, adapt, and spread.

Future threats are difficult to predict but will certainly include the increasingly complex challenges of foodborne and waterborne diseases, the threat of another influenza pandemic, emerging antibacterial and antiviral resistance, and the likelihood of increasing problems with zoonotic diseases. What new diseases will be encountered in the next 20 years? What role will the increasingly advanced field of molecular biology play? Will other infectious agents from the past, in addition to the 1918 influenza virus, be resurrected? Or will increasingly advanced bioterrorists or rogue nations be able to create the ultimate weapons through genetic engineering? Meeting these challenges will require continued research with a multidisciplinary approach, using the expertise of physicians and veterinarians trained in public health, microbiologists, pathologists, ecologists, vector biologists, and public health officials, both military and civilian.

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ABBREVIATIONS AND ACRONYMS

A

AAALAC: Association for Assessment and Accreditation of Laboratory Animal Care
ABSA: American Biological Safety Association
ABSL: animal biosafety level
ACC: acute care center
ACIP: Advisory Committee for Immunization Practices
AFBAT: Air Force biological augmentation team
Agr: accessory gene regulator
AHF: Argentine hemorrhagic fever
AIDS: acquired immunodeficiency syndrome
AML: Army medical library
AMPA: alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APACHE: Acute Physiology and Chronic Health Evaluation
APHIS: Animal and Plant Health Inspection Service
APHL: Association of Public Health Laboratories
AR: Army regulation
ASP: amnesic shellfish poisoning
ATLS: advanced trauma life support
AVA: anthrax vaccine adsorbed
AWR: animal welfare regulation

B

BIDS: Biological Integrated Detection System
BMBL: Biosafety in Microbiological and Biomedical Laboratories
BPRP: biological personnel reliability program
BSAT: biological select agents and toxins
BSC: biological safety cabinet
BSE: bovine spongiform encephalopathy
BSL: biosafety level
BT: bioterrorism
BW: biowarfare
BWC: Biological Weapons Convention

C

CBC: complete blood count
CBIRF: Chemical-Biological Incident Response Force
CBRNE: chemical, biological, radiological, nuclear, or high-yield explosive
CBSP: Certified Biological Safety Professional
CCHF: Crimean-Congo hemorrhagic fever
CDC: Centers for Disease Control and Prevention
CEV: cell-associated virion
CFR: Code of Federal Regulations
CFT: complement fixation test
CFU: colony-forming unit
CHA: cystine heart agar
CHOC: chocolate agar
cipro: ciprofloxacin
CLSI: Clinical Laboratory Standards Institute
CMV: cytomegalovirus
CNS: central nervous system
CO: certifying official
CONUS: continental United States
CSF: cerebrospinal fluid
CTS: Casualty Transportation System
CXR: chest x-ray

D

DA: Department of the Army
DAD: diode array detection
dB: decibel

DC: dendritic cell
DCO: defense coordinating officer
DELFLIA: dissociation-enhanced lanthanide fluorescence immunoassay
DFA: direct fluorescent antibody
DHF/DSS: dengue hemorrhagic fever/dengue shock syndrome
DHHS: Department of Health and Human Services
DHS: Department of Homeland Security
DIC: disseminated intravascular coagulation
DNA: deoxyribonucleic acid
dNTPs: deoxyribonucleotide triphosphates
DoD: Department of Defense
doxy: doxycycline
DSS: dengue shock syndrome

E

ECL: electrochemiluminescence
EEE: Eastern equine encephalitis
EEV: extracellular enveloped virion
EF: edema factor
ELISA: enzyme-linked immunosorbent assay
EM: electron microscopy
EMS: emergency medical services
EPA: US Environmental Protection Agency
ER: endoplasmic reticulum
ESF: emergency support function
ESSENCE: Electronic Surveillance System for the Early Notification of Community-based Epidemics

F

F1: fraction 1
FAD: foreign animal disease
FBI: Federal Bureau of Investigation
FCO: federal coordinating officer
FDA: Food and Drug Administration
FDP: fibrin degradation product
FDPMU: forward deployable preventive medicine unit
FEMA: Federal Emergency Management Agency
FHP: Force Health Protection
FMD: foot and mouth disease
FRET: Fluorescence Resonance Energy Transfer

G

GCP: Good Clinical Practices
GLC: gas-liquid chromatography
GP: glycoprotein
GS: Government Schedule

H

H: hour
HA: hemagglutinin
HAZMAT: hazardous material
HEPA: high-efficiency particulate air
HF: hemorrhagic fever
HFRS: hemorrhagic fever with renal syndrome
HIV: human immunodeficiency virus
HLA: human leukocyte antigen
HPLC: high-performance liquid chromatography
HPLC-MS: high-performance liquid chromatography-mass spectrometry
HPS: hantavirus pulmonary syndrome
HUS: hemolytic uremic syndrome

I

ICAM: intercellular adhesion molecule
ICS: Incident Command System
IEV: intracellular enveloped virion
IFN: interferon
IgG: immunoglobulin
IL: interleukin
IM: intramuscular
IMP: inosine-5'-phosphate
IMV: intracellular mature virion
IND: investigational new drug
IRB: institutional review board
IV: intravenous

J

JBAIDS: Joint Biological Agent Identification and Diagnostic System
JBPDS: Joint Biological Point Detection System
JCAHO: Joint Commission on the Accreditation of Healthcare Organizations
JE: Japanese encephalitis
JFO: Joint Field Office
JTF-CS: Joint Task Force for Civil Support

K

Kb: kilobase
kd: kilodalton

L

LCR: low calcium response
LCV: large cell variant
LD: lethal dose
LF: lethal factor
LLAP: Legionella-like amoebal pathogen
LPS: lipopolysaccharide
LRN: Laboratory Response Network
LVS: live vaccine strain

M

MA: microagglutination titer
MAPK: mitogen-activated protein kinase kinase
MASCAL: medical mass casualty event
Mb: megabase
MCC: Medical Command and Control
MEMS: Modular Emergency Medical System
MGBP: Minor Groove Binding Protein
MHC: major histocompatibility complex
MIC: mean inhibitory concentration
MOPP: military-oriented protective posture
MOU: Memorandum of Understanding
MRSA: methicillin-resistant *Staphylococcus aureus*
MS: mass spectrometry
MSDS: material safety data sheet
MVA: Modified Vaccinia Ankara

N

NA: neuraminidase
NA: not applicable or not done
NACLC: National Agency Check, Local Agency Check, and Credit Check
NCCLS: National Committee for Clinical Laboratory Standards
NDMS: National Disaster Medical System
NEHC: Neighborhood Emergency Help Center
NIH: National Institutes of Health

NIMS: National Incident Management System
NIOSH: National Institute for Occupational Safety and Health
NIV: nivalenol
NL: normal limit
NO: nitric oxide
NORTHCOM: US Northern Command
NP: nucleoprotein
NPT: National Pipe Thread
NRM: National Registry of Microbiologists
NRP: National Response Plan
NSP: neurotoxic shellfish poisoning
NYC: New York City

O

OIE: Office International des Epizooties
OSHA: Occupational Safety and Health Administration

P

PA: protective antigen
PAPR: powered air-purifying respirator
PBT: pentavalent botulinum toxoid
PC: percutaneous
PCR: polymerase chain reaction
PDI: potentially disqualifying information
PDI: protein disulfide isomerase
Pfu: plaque-forming unit
Pla: plasminogen activator
PO: by mouth
POD: point of distribution
ppb: part per billion
PPE: personal protective equipment
ppm: part per million
PRN: as needed
PRNT: plaque reduction neutralization titer
PSP: paralytic shellfish poisoning

Q

Q: each, every
QD: every day

R

RANTES: regulated upon activation, normal T-cell expressed and secreted
RAPID: Ruggedized Advanced Pathogen Identification Device
RBP: Registered Biosafety Professional
RDTE: research, development, test, and evaluation
RFLP: restriction fragment-length polymorphism
RIP: ribosome-inactivating protein
RODS: Realtime Outbreak Detection System
RSV: respiratory syncytial virus
RT-PCR: reverse transcriptase polymerase chain reaction
RVF: Rift Valley fever
rx: prescription

S

SAF: Singapore Armed Forces
SARS: severe acute respiratory syndrome
SBA: sheep blood agar
SCO: state coordinating officer
SCV: small cell variant
SE: staphylococcal enterotoxin
SEB: staphylococcal enterotoxin B
SERPACW: skin exposure reduction paste against chemical warfare agents
sGP: secreted glycoprotein

SNS: strategic national stockpile
SOP: standard operating procedure
SPE: streptococcal pyrogenic exotoxin
SQ: subcutaneous
STARI: southern tick-associated rash illness
STX: saxitoxin

T

TB: tuberculosis
TBE: tick-borne encephalitis
TC: cell culture
TCID: tissue culture infecting dose
TF: tissue factor
TIGER: triangulation identification for genetic evaluation of risks
TK: thymidine kinase
TLC: thin-layer chromatography
TLR: Toll-like receptor
TMP-SMZ: trimethoprim-sulfamethoxazole
TRAIL: tumor necrosis factor-related, apoptosis-inducing ligand
TrD: Trinidad donkey
TRF: time-resolved fluorescence
TSS: toxic shock syndrome
TSST-1: toxic shock syndrome toxin

U

UN: United Nations
UNSCOM: United Nations Special Commission on Iraq
USA: United States of America
USAMRICD: US Army Medical Research Institute of Chemical Defense
USAMRIID: US Army Medical Research Institute of Infectious Diseases
USAMRMC: US Army Medical Research and Materiel Command
USDA: US Department of Agriculture

V

V: virulence
VALTrex: valacyclovir hydrochloride
VEE: Venezuelan equine encephalitis
VHF: viral hemorrhagic fever
VIG: vaccinia immune globulin
VLS: vascular leak syndrome
VP: virion protein
VRE: vancomycin-resistant enterococci

W

WBC: white blood cell
WEE: Western equine encephalitis
WHO: World Health Organization
WNV: West Nile virus

Y

YOPS: yersinia outer protein

INDEX

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