Chapter 23

Defense Against Biological Weapons (Biodefense)

Biological warfare (germ warfare) is defined as the use of any disease-causing organism or toxin(s) found in nature as weapons of war with the intent to destroy an adversary. Though rare, the use of biological weapons has occurred throughout the centuries.

The ban on the use of biological weapons was enacted as an international law by the Geneva Protocol of 1925. Subsequently, because a successful biological attack could conceivably result in thousands, even millions, of casualties, causing severe disruptions to societies and economies, the creation and stockpiling of biological weapons were banned in 1972 by the Geneva Biological and Toxin Weapons Convention, which was signed by more than 100 states. The convention extended the ban to almost all production, storage, and transport of biological weapons. *However, oddly enough, the convention did not prohibit the use of biological weapons*. The consensus among military experts is that, except in the context of bioterrorism, biological warfare is militarily of little use (http://www.biocrawler.com/encyclopedia/biological_warfare). It is believed that since the signing of the convention, the number of countries capable of producing such weapons has increased.

For the military, the main problem with use of biological weapons is that a biological attack would take some time to have an effect and, therefore, unlike a nuclear or chemical attack, would not necessarily stop an advancing army. As a strategic weapon, biological warfare would again be militarily problematic, because unless it is used to poison the enemy's civilian population, it would be difficult to prevent the biological attack from spreading, either to allies or to the attacker. Besides, a biological attack would almost certainly invite immediate massive retaliation, usually in the same form (http://www.biocrawler.com/encyclopedia/biological_warfare).

On the other hand, a terrorist attack using biological agents, once thought to be a remote possibility, occurred in autumn 2001 when *B. anthracis* spores were sent through the U.S. mail, causing 18 confirmed cases of anthrax (11 inhalation, 7 cutaneous). Recent events have raised awareness of both the possibility of a bioterrorist attack and the vulnerability of the U.S. population to such an event. In 2003 and 2004, ricin was found in an envelope at a postal facility in South Carolina and in a U.S. Senate office building in Washington, DC, and was used to contaminate several jars of baby food in California. Although the U.S. Department of Defense (DoD) has developed defenses for biological warfare, there are additional concerns that need to be addressed to provide an adequate civilian defense from a bioterrorist attack. The potential list of microbial pathogens that threaten civilian populations is larger than the list of classic biological warfare threats. The list of biodefense priority pathogens targeted by NIAID research can be found at http://www3.niaid.nih.gov/Biodefense/bandc_priority.htm. In addition, the populations to be protected are different, because civilians include people of all ages and physical conditions.

Diseases considered for weaponization, or known to be weaponized, include anthrax, Ebola, bubonic plague, cholera, tularemia, brucellosis, Q fever, Machupo, Venezuelan equine encephalomyelitis (VEE), and smallpox. Naturally occurring toxins that can be used as weapons include ricin, staphylococcal enterotoxin B (SEB), the botulism toxin, and many mycotoxins.

Instead of targeting humans, biological weapons could be designed to target food crops. Such bioweapons are known as bioherbicides, or mycoherbicides if the agent is a fungus.

23.1 History of Biological Warfare

The use of biological agents in military confrontations is not new and before the 20th century occurred in three main forms:

- *Deliberate poisoning of food and water with infectious material*. For example, in the 6th century BC, the Assyrians poisoned enemy wells with a fungus that would make the enemy delusional. During the U.S. Civil War, General George Sherman reported that Confederate forces shot

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farm animals in ponds upon which the Union forces depended for drinking water, thus effectively poisoning the water (http://www.biocrawler.com/encyclopedia/biological_warfare).

- *Use of microorganisms, toxins, or animals, living or dead, in a weapons system*. During the Middle Ages, victims of bubonic plague were used for biological attacks, often by flinging their corpses and excrement over castle walls using catapults *(1)*. In medieval medical theory, the stench of rotting organic material was believed to be a potent cause of disease. Thus, in 1340, the enemy hurled dead horses and other animals by catapult at the castle of Thun L'Eveque in Hainault (now in Northern France). At Caffa in the Crimea in 1346, it was human plague cadavers, and in Karlstein in Bohemia in 1422, it was human battle casualties and waste of some kind—probably human and animal manure. The purpose was almost certainly to transmit disease, and at least in 1346 at Caffa it succeeded: after the battle a large number of the defenders came down with plague, and by fleeing Caffa they helped transmit the disease around the Mediterranean basin, initiating the Black Death *(1)*.

The last known incident of using plague corpses for biological warfare occurred in 1710, when Russian forces attacked the Swedes by flinging plague-infected corpses over the city walls of Reval.

- *Use of biologically inoculated humans and fabrics*. Much of the Native American population suffered after contact with the Old World as a result of the introduction of many different diseases. The British army at least once used smallpox as a weapon. In 1763, at Fort Pitt on the Pennsylvania frontier during the parlay, the British gave as gifts to the Indians blankets and handkerchiefs that were taken from smallpox patients in the infirmary *(1)*.

During the American Revolutionary War, Britain apparently used smallpox as a biological weapon, possibly on several occasions *(1, 2)*. In the northern colonies they were suspected on several occasions of inoculating civilians with smallpox with the intent that they would transmit the disease to the Continental Army *(1)*. Deliberate inoculation with material from a small pustule was a well-known protective measure: it gave the recipient a mild case of smallpox, with lower chance of death than that with natural transmission and led to lifelong immunity. However, the induced disease was as contagious as natural smallpox, and the inoculated persons were commonly quarantined until their symptoms abated.

23.1.1 German Biological Sabotage in World War I

From 1915 through 1918, Germany waged a campaign of covert biological attack on animals (horses, mules, sheep, and cattle) being shipped from neutral countries to the Allies *(3)*. For biological weapons, the program used glanders and anthrax and employed secret agents to administer the bacterial cultures to animals penned for shipment from Romania, the United States, Spain, Argentina, and perhaps Norway. It is not clear how effective these programs of biological sabotage were.

23.1.2 Japanese Biological Warfare in World War II

Contrary to expectations, in fact only Japan made significant use of biological weapons during World War II *(4, 5)*. During 1939, the Japanese carried out biological attacks on military and civilian targets. The methods were primitive, and all efforts to develop reliable and effective biological munitions failed. Most attacks relied on saboteurs contaminating wells with intestinal pathogens and on distribution of microbe-laced foods, air drops of plague-infected fleas, and probably aerial spraying of microbial cultures. Their effectiveness is hard to evaluate.

23.1.3 Terrorist Use of Biological Weapons

In 1984, followers of the Bagwan Shree Rajneesh (the Rajneeshee cult) living in a ranch in rural Oregon tested a crude biological weapon in the small town of The Dulles *(6)*. Cultures of *Salmonella typhimurium* were grown in the infirmary of the ranch and then sprinkled on food in restaurants, in particular at salad bars. The result was more than 750 cases of salmonellosis, 45 of which required hospitalization. The intent of the attack, which was apparently successful, was to determine the feasibility of keeping voters from the polls in an upcoming election, with the hope that the Rajneeshees could win a majority in the county government, allowing them to make changes in the zoning and land-use policies that the existing government had turned down *(1)*.

During September and October 2001, several letters (probably five to six) containing *Bacillus anthracis* spores were mailed from Trenton, New Jersey, to several media representatives and to two offices at the U.S. Senate *(1)*. As these envelopes were processed through automatic

sorting machines, they contaminated a great number of postal workers, and upon being opened, they contaminated hundreds more people in the receiving premises. A total of 22 cases of anthrax are thought to have resulted (a few more are possible). Eleven of the episodes were cutaneous, none of which was fatal. The other cases were pulmonary, and five of them were fatal. The widespread and extensive use of prophylactic antibiotic treatment undoubtedly prevented many additional cases of infection.

23.2 Biological and Toxin Weapons Factors

Delivery of biological weapons through food or water is of concern. However, it is restricted by the quantity of biological agent required, which limits use to objectives where less than mass morbidity is intended (e.g., psychological impact on civilian populations) *(7)*. Contrary to popular perception, dilution factors and modern technology for refining the food supply, including water purification, will significantly limit the efficient use of biological weapons by the oral use of exposure *(8)*. By comparison, in the context of biological warfare and/or terrorist action, biological weapons are most likely to be delivered covertly and by aerosol, with the intention of causing mass casualties *(7)*.

Potential biological weapons are usually designated as either lethal or incapacitating, although these terms are not absolute but imply statistical probability of response.

In April 1997, the U.S. Department of Health and Human Services (DHHS) and the Centers for Disease Control and Prevention (CDC) set forth a set of regulations governing hazardous biological agents (Tables 23.1 and 23.2) *(9)*.

23.2.1 Aerosol Delivery: Biological/Toxin Agent Factors

Biological weapons can be disseminated most effectively in an aerosol form through the use of either conventional delivery systems, such as bombs and missiles, or by unconventional systems, such as agricultural sprayers. Either way, respirable aerosols represent the most serious threat of effectively exposing large numbers of people *(7)*.

Factors that can influence the effectiveness of aerosol delivery of potential biological/toxin agents include infectivity, virulence, toxicity, pathogenicity, incubation period, transmissibility, lethality, and stability *(7, 10)*.

Infectivity. The capability of microorganisms to establish themselves in the host environment differs. Whereas pathogens with high infectivity cause disease with relatively few organisms, those with low infectivity will require a larger number. To this end, high infectivity does not necessarily imply that the symptoms of disease will appear more quickly or that the disease will be more severe *(7)*.

Virulence. The propensity of an agent for causing severe disease is dependent on a diverse combination of agent and host factors. These factors are dynamic and malleable, leading often different strains of the same microorganisms to cause diseases with different severity *(7)*.

Toxicity. The toxicity relates to the severity of the illness (toxicosis) elicited by the toxin *(7)*.

Pathogenicity. Pathogenicity is defined as the ability of an agent to initiate a set of events (e.g., propagate attachment and penetration) that will culminate in a disease or abnormality. In this regard, a sufficient number of microorganisms or quantity of toxin must penetrate the host to initiate an infection (the infective dose) or intoxication (the intoxicating

Table 23.1 The CDC list of restricted agents, 1997

Viral	Bacterial	Toxins	Rickettsial	Fungal
Crimean-Congo hemorrhagic fever	*Bacillus anthracis*[1]	Abrin	*Coxiella burnetii*[1]	*Coccidioides immitis*
Eastern equine encephalitis virus	*Brucella abortus, melitensis,* and *suis*[1]	Aflatoxins	*Rickettsia prowazekii*	
Ebola virus[1]	*Burkholderia* (*Pseudomonas mallei*)[1]	Botulinum neurotoxins[1]	*Rickettsia rickettsii*	
Equine morbillivirus	*Burkholderia* (*Pseudomonas pseudomallei*)[1]	*Clostridium perfringens* epsilon toxin[1]		
Lassa fever virus	*Clostridium botulinum*	Conotoxins		
Marburg virus[1]	*Francisella tularensis*[1]	Diacetoxyscirpenol		
Rift Valley fever virus	*Yersinia pestis*[1]	Ricin[1]		
South American hemorrhagic fever viruses		Saxitoxin		
Tick-borne encephalitis complex viruses		Shigatoxin		
Variola major virus (smallpox virus)[1]		*Staphylococcus enterotoxins*[1]		
Venezuelan equine encephalitis virus[1]		Tetradotoxin		
Hantavirus pulmonary syndrome		T-2 toxin (trichothecene)[1]		
Yellow fever virus				

[1]Based on various assumptions and estimations as discussed in Section 23.2.1, these agents possess the attributes that make them candidates for biological aerosol weapons affecting large populations [with permission *(7)*].

Table 23.2 Emergency preparedness and response: Bioterrorism agents/diseases by categories

Category A[1]	Category B[2]	Category C[3]
Anthrax (*Bacillus anthracis*)	Brucellosis (*Brucella* spp.)	Nipah virus
Botulism (*Clostridium botulinum* toxin)	Epsilon toxin (*Clostridium perfringens*)	Hantavirus
Plague (*Yersinia pestis*)	Food safety threats (e.g., *Salmonella* spp., *Escherichia coli* 0157:H7, *Shigella*)	
Smallpox (variola major)	Glanders (*Burkholderia mallei*)	
Tularemia (*Francisella tularensis*)	Melioidosis (*Burkholderia pseudomallei*)	
Viral hemorrhagic fevers [filoviruses (e.g., Ebola, Marburg) and arenaviruses (e.g., Lassa, Machupo)]	Psittacosis (*Chlamydia psittaci*)	
	Q fever (*Coxiella burnetii*)	
	Ricin toxin from *Ricinus communis* (castor beans)	
	Staphylococcal enterotoxin B	
	Typhus fever (*Rickettsia prowazekii*)	
	Viral encephalitis [alphaviruses (e.g., Venezuelan equine encephalitis, eastern equine encephalitis, western equine encephalitis)]	
	Water safety threats (e.g., *Vibrio cholerae, Cryptosporidium parvum*)	

[1]High-priority agents because they (i) can be easily disseminated or transmitted from person to person; (ii) result in high mortality rates and have the potential for major public health impact; (iii) might cause public panic and social disruption; and (iv) require special action for public health preparedness.
[2]Second highest priority agents/toxins, including those that (i) are moderately easy to disseminate; (ii) result in moderate morbidity rates and low mortality rates; and (iii) require specific enhancements of CDC's diagnostic capacity and enhanced disease surveillance.
[3]Third highest priority agents, including emergency pathogens that could be engineered for mass dissemination in the future because of (i) availability; (ii) ease of production and dissemination; and (iii) potential for high morbidity and mortality rates and major health impact.

dose). Infectious agents must then multiply (replicate) to produce disease *(7)*.

Incubation Period. Incubation period is the time between exposure and the appearance of symptoms of disease. This period depends on many variables, including the initial dose, virulence, route of entry, rate of replication, and host immunologic factors *(7)*.

Transmissibility. Some biological agents can be transmitted from person-to-person directly. However, indirect transmission such as by arthropod vectors may also be a significant means of transmission and spread of disease to humans. With regard to managing casualties in biological warfare, the relative ease with which an agent is passed from person to person (i.e., its transmissibility) constitutes the principal concern *(7)*.

Lethality. Lethality reflects the relative ease with which an agent causes death in a susceptible population.

Stability. The viability of an agent is affected by various environmental factors, including temperature, relative humidity, atmospheric pollution, and sunlight. A quantitative measure of stability is an agent's decay rate (e.g., "aerosol decay rate") *(7)*.

23.3 Anthrax (Bacillus anthracis)

Anthrax is caused by *Bacillus anthracis*. A disease in animals, anthrax can be transmitted to humans. Because *B. anthracis* forms spores that can be aerosolized and sprayed with the intent to kill, this pathogen represents a very serious threat as an agent of biological warfare and bioterrorism *(11, 12)*. From 1979 to 1985, a large outbreak (about 10,000 cases) occurred in Zimbabwe—at that time, a civil war caused the interruption of veterinary and public health services in the country, which contributed significantly to the magnitude of the outbreak *(11)*. This, and the accidental release of spores into the air in Sverdlovsk (in the former Soviet Union), and the recent mail attacks in the United States in 2001 that led to human casualties, became a stark reminder of the pathogenic potential of *B. anthracis* as a biological weapon. Furthermore, for some time, *B. anthracis* has been the subject of intense research and genetic manipulation with the intent of generating pathogen variants with increased virulence or with resistance to medical therapies and vaccine prevention strategies *(4, 11–13)*.

B. anthracis can be obtained from infected animals or soil, and spores from the pathogen can be easily prepared. The anthrax spores usually display very low visibility when delivered as an aerosol spray or powder. The lethal dose (LD_{50}) after human inhalation of the spores is not known, but when estimated from animal studies it is of the order 10,000 spores, corresponding to approximately 0.01 μg *(14)*.

23.3.1 Bacteriology of B. anthracis

B. anthracis was shown to be the etiologic agent of anthrax by Robert Koch *(15)*. Its name derived from the Greek word for *coal* (*anthracis*), because of the black skin lesions that arise during the course of cutaneous infections.

B. anthracis is a Gram-positive, aerobic, spore-forming bacillus *(15)*. The vegetative cell is rather large (1 to 8 μm long, and 1.0 to 1.5 μm wide), whereas the spore size is approximately 1.0 μm. The bacilli are nonmotile and form large colonies with irregularly tapered outgrowths.

When ingested by herbivores, *B. anthracis* germinate within the host as they enter an environment rich in amino acids, nucleosides, and glucose. The vegetative bacilli multiply rapidly in the host and express virulence factors that kill the host. Then they sporulate in the cadaver once in contact with air and then contaminate the soil, anticipating another host. Vegetative bacilli have poor survival outside of an animal or human host. For example, when inoculated into water, colony counts will decline rapidly within 24 hours *(11)*.

23.3.2 Pathogenesis of B. anthracis

The vegetative form of *B. anthracis* elaborates a capsule that confers an antiphagocytic property on the bacilli *(16, 17)* and secretes the three-component protein toxin, PA (*pagA* encoded), LF (*lef* encoded), and EF (*cya* encoded) *(18)*. The combined action of the protein toxins is believed to kill infected animals by triggering the release of interleukin (IL-1) and tumor necrosis factor-α from the intoxicated macrophages *(19)*. The two binary exotoxins are referred to as LeTx when comprising PA and LF and as EdTx when encompassing PA and EF *(11)*.

LeTx has been implicated in both macrophage and host death *(20, 21)*. It acts as a zinc protease and cleaves the mitogen-activated protease kinase kinase (MAPKK), presumably interfering with signal transduction events of the p38 pathway and causing apoptosis of activated macrophages and release of IL-1 cytokine *(20, 22)*.

EdTx is thought to be involved in phagocyte inhibition and the massive edema occurring during anthrax infection *(23)*. EF is an adenylate cyclase *(24)*. After binding to calmodulin, EdTx cleaves the adenosine triphosphate (ATF) to generate the second messenger cyclic adenosine monophosphate (cAMP), thereby presumably interfering with the normal immune function of the macrophages *(24)*. At this stage, *B. anthracis* will multiply in the lymph system and in the blood but not within the macrophages *(11)*.

23.3.2.1 Virulence Plasmids

The virulence of all *B. anthracis* strains requires two large plasmids, pXO1 and pXO2 *(25,26)*. pXO1 carries the genetic determinants responsible for the synthesis of the anthrax exotoxin complexes PA, LF, and EF *(27)*, whereas pXO2 is involved in the capsule production *(28)*.

23.3.3 Human Disease

Humans are accidental hosts of *B. anthracis*. Infection is initiated with the introduction of spores into skin lesions, or entry through the intestinal or respiratory mucosa. Depending on the route of entry, anthrax could be classified as either cutaneous, gastrointestinal, or inhalational *(11, 29)*.

Humans acquire anthrax infections from contact with infected animals or contaminated animal products (e.g., hides, wool, hair, ivory tusks). Ingestion of poorly cooked infected meat may lead to gastrointestinal anthrax. Cases of inhalational anthrax have been observed in individuals who process animal products, and where aerosolized spores may be inhaled *(11)*.

23.3.3.1 Inhalational Anthrax

Inhalation of spores by humans will cause bacterial germination in the hilar and mediastinal lymph nodes *(29)*. The classic clinical description of inhalational anthrax is that of a biphasic illness. After an incubation period of 1 to 6 days, mild fever, malaise, myalgia, nonproductive cough, and some chest and abdominal pain may be observed *(11)*. As the disease develops further, fever, acute dyspnea, diaphoresis, and cyanosis will occur. The entry of the pathogen into the bloodstream will lead to systemic spread to the intestines and the meninges. During the meningitis, the cerebrospinal fluid is hemorrhagic with polymorphonuclear pleocytosis *(11)*.

23.3.3.2 Cutaneous Anthrax

In cases of cutaneous anthrax, spores of *B. anthracis* will enter through a small abrasion or wound in the human skin, typically in the face, hands, or neck *(29)*. Cutaneous anthrax represents 95% of all naturally occurring anthrax. The primary lesion, a pruritic papule, appears within a few days (1 to 7 days). Then, it develops into an ulcer with surrounding vesicles; occasionally, a single larger vesicle will form (1 to 2 cm in diameter) *(29)*. The vesicle is filled with clear or serosanguineous fluid containing occasional leukocytes and numerous large bacilli. After 2 days, the vesicle will rupture and will undergo necrosis, and a painless characteristic black eschar with a surrounding edema can be observed. After 1 to 2 weeks, the lesion will dry and the eschar separates, revealing an underlying scar. Systemic infections are almost always

lethal if left untreated *(11)*. Antibiotic therapy will not prevent the progression of skin lesions but will abbreviate or prevent systemic infection.

23.3.3.3 Gastrointestinal Anthrax

Consumption of food that is contaminated with *B. anthracis* spores will lead to gastrointestinal anthrax *(29)*. The characteristic skin lesion is not present, and the establishment of a definitive diagnosis outside of an epidemic is difficult *(11)*. The incubation period of gastrointestinal anthrax is only 3 to 7 days. Abnormal symptoms with nausea, vomiting, anorexia, and fever may develop. The disease manifestations will progress rapidly, and patients will present with severe, bloody diarrhea. The primary intestinal lesions are ulcerative and occur mainly in the terminal ileum or cecum. Hemorrhagic mesenteric lymphadenitis is also a feature of gastrointestinal anthrax, and marked ascites may occur. The mortality associated with gastrointestinal anthrax is greater than 50%, and death can occur within 2 to 5 days after the onset of symptoms *(11)*.

23.3.3.4 Treatment of Anthrax

With the availability of antibiotics, these agents became the preferred drugs of treatment *(30)*. Successful therapy usually relies on the prompt uptake of antibiotics. Chemotherapy is rarely effective in cases of inhalational anthrax because the exposure to spores may not be recognized for some time. In this regard, the first stages of the illness are often mistaken for bronchitis and the second stage for cardiac failure or cerebrospinal accidents. Spores engulfed by alveolar macrophages are carried to local lymph nodes, germinate, and will rapidly multiply in the bloodstream *(11)*.

During the anthrax attack in the United States in 2001, the antimicrobial susceptibility patterns of all *B. anthracis* isolates displayed susceptibility to ciprofloxacin, doxycycline, chloramphenicol, clindamycin, tetracycline, rifampin, vancomycin, penicillin, and amoxicillin *(31)*. Because of the mortality associated with inhalational anthrax, CDC-issued guidelines recommended that two or more antimicrobial agents be used for effective treatment and that ciprofloxacin or doxycycline be used in an initial intravenous therapy. The duration of the therapy should be prolonged over 60 days *(31)*. Treatment of systemic anthrax using penicillin alone is no longer recommended, because *B. anthracis* genome encodes for at least two β-lactamases: a penicillinase and a cephalosporinase *(31)*.

However, whereas the currently available antibiotic treatments are capable of killing multiplying bacteria, they are unable to interfere with the toxin-mediated killing of the host. Another avenue to be explored is the possibility of combining passive immunization of infected patients with aggressive antibiotic treatment. Most attempts described to date have been directed at interfering with the correct functioning of the PA exotoxin subunit. However, no matter how effective a vaccine may be, multiple immunizations would be required for protection to build up *(11)*.

Studies on the *anthrax toxin receptor (ATR)*, a recently discovered membrane protein on the surface of macrophages *(32)*, have demonstrated that the soluble region of ATR can block the killing by PA, suggesting that the design of a receptor decoy may represent a new therapeutic strategy. However, because the physiologic function of ATR (TEM8) is not known, it is unclear whether such a therapeutic agent (a receptor decoy) will also affect the host *(11)*.

Another strategy for blocking the function of PA is to take advantage of mutant PA exhibiting a dominant negative phenotype. Such mutant PAs when co-assembled with the wild-type protein in a test tube prevented pore formation and translocation of EF and LF into cultured cells. Injection of the mutant PA in rats challenged with anthrax conferred protection from disease *(33)*. It is conceivable that administration of mutant PA into individuals infected with anthrax may block the progression of the disease at even more advanced stages of the disease *(11)*.

Recently, in a new therapeutic approach, the inherent binding specificity and lytic action of the bacteriophage enzymes, *lysins*, has been explored *(34)*. As a normal part of their life cycle, bacteriophages multiply in host cells and their progeny are released upon lysis of the cell wall. Purified PlyG, an enzyme synthesized by bacteriophage-γ, was found to destroy the cell wall of both vegetative cells and germinating spores of *B. anthracis (34)*. PlyG is a specific lysine for *B. anthracis* and other members of the *B. anthracis* “cluster” of bacilli and may well be exploited as a tool for treating and detecting anthrax.

The *Anthrax Vaccine Absorbed (AVA)* is an adjuvant absorbed preparation of the culture supernatant of vaccine strains and has been licensed by the Food and Drug Administration (FDA) for the prevention of anthrax in humans *(35)*. The AVA vaccine has been successfully used to prevent laboratory infections in the United States, and its effectiveness in preventing anthrax after a respiratory challenge has been demonstrated in rodents and non-human primates *(36)*. The active component of the AVA vaccine, anti-PA IgG, is believed to exert sporicidal effect. However, the effectiveness of the AVA vaccine in preventing human anthrax after a bioterrorist attack or biological warfare use of *B. anthracis* is hitherto unknown *(11)*.

23.4 Variola Major Virus (Smallpox)

The month of October 1977 will be remembered as the time when the world's last acquired case of smallpox occurred in Somalia, Africa *(37)*. The last case of smallpox (a laboratory-associated infection) occurred in England in September of 1978. After a worldwide program to verify the eradication of smallpox in May 1980, the 33rd World Health Assembly accepted the report of the Global Commission for the Certification of Smallpox Eradication that smallpox had been eradicated *(38)*. However, even though smallpox had been eliminated as a threat to humanity as the result of worldwide efforts involving intensive planning, geopolitical cooperation, and effective vaccination, it still remains a great public health concern. Although two WHO-approved repositories of variola virus remain at the CDC in Atlanta, Georgia, and at "Vector" in Novosibirsk, Russia, the extent of concealed stockpiles in other parts of the world is unknown. The former Soviet Union has admitted to weaponizing variola major *(39)*.

Until its eradication in 1980, smallpox was one of the most dangerous infectious diseases, responsible over the centuries for more deaths and disabilities than any other pathogen *(37)*. Those fortunate to survive the infection often faced lifelong complications and sequelae such as blindness, arthritis, encephalitis, permanent osteoarticular anomalies, and severely scarred complexion *(40)*.

It is thought that the variola virus evolved as a human pathogen from an Orthopoxvirus of animals in the Central African rainforests and established itself in the valley of the Nile and the Fertile Crescent of Egypt thousands of years ago *(37)*. Examination of Egyptian mummified remains demonstrated the presence of typical pustular eruptions of smallpox *(41)*.

Before the development of the cowpox vaccine by Dr. Edward Jenner in 1796, there was no safe and consistently effective prevention method for smallpox *(42)*.

Global terrorism, the continued research and development of biological weapons, and other geopolitical events have generated a concern that smallpox could be directed at susceptible populations *(38)*. Even if the legally sanctioned repositories of smallpox in the United States and Russia were to be destroyed, other potential sources of smallpox could exist, including clandestine stockpiles, cadavers in permafrost, and strains reverse-engineered by scientists using data obtained from genetically sequenced strains. In fact, it is generally agreed that the likelihood of smallpox virus existing outside WHO-sanctioned laboratories is high *(38)*.

These and other findings have led the U.S. federal authorities to conclude that the threat of smallpox as a biological weapon is significant enough to warrant reinstituting a vaccination program for selected members of the U.S. population, including the military, health care workers, and certain emergency responders, as well as the possibility of making the vaccine available to the general population on a volunteer basis in the future *(43)*.

23.4.1 Variola Virus

The variola virus is a member of the family Poxviridae, subfamily Chordopoxvirinae, genus Orthopoxvirus. The genus Orthopoxvirus comprises many related viruses and includes, in addition to variola major, vaccinia virus, cowpox, monkeypox, ectromelia (mousepox), camelpox, taterapox (gerbilpox), Uasin Gishu disease, and racoonpox *(38)*. The variola virus is exclusively a human disease, with no known animal reservoir. Other members of the genus produce disease primarily in their respective animal hosts. However, because of the close relationships between the orthopoxviruses, species jumping can occur, as evidenced by cowpox and monkeypox; monkeypox is capable of producing large outbreaks of human disease *(44, 45)*, with clinical manifestations very similar to those of variola major and a mortality rate of 10% to 15% *(44)*.

23.4.1.1 Morphology and Antigenic Structure of Orthopoxviruses

Poxviruses are the largest of all viruses, with a genome consisting of a single molecule of double-stranded DNA cross-linked with a hairpin loop at the ends *(38)*. Their genomes vary in size from 130 to 300 kbp *(46)*. The physical properties of the poxviruses are very similar in that they are "brick-shaped" or ovoid, and are 200 to 400 nm long. The external surface of the virus contains an envelope consisting of host cell membrane protein plus virus-specific polypeptides such as hemagglutinin. Based on studies of vaccinia virus, the presence or absence of an outer envelope defines two major infectious forms of the virus: the intracellular mature virion (IMF) and the extracellular enveloped protein (EEV) *(47)*. The IMF form is stable in the environment and plays a predominant role in the host-to-host transmission. The EEV form plays an important role in dissemination within the host. Below the envelope is the outer membrane, consisting of a complex of surface tubules and globular proteins and enclosing large lateral bodies. The viral core consists of double-stranded DNA that is unique to each virus as shown by restricted fragment length polymorphism (RFLP) analysis; the genomes of the orthopoxviruses have a very well-preserved central region, allowing classification within the genus, whereas heterology in the terminal regions can be used to differentiate species *(38)*.

The antigenic structure of the orthopoxviruses is characterized by large number of polypeptides that define cellular receptors and antigenic sites for protective immunity. The close relations of these viruses (e.g., vaccinia virus and variola virus) and serologic cross-reactivity help to explain the importance of the type of antigen required for protective immunogenicity. Thus, mice vaccinated with VACV L1R (IMV immunogen) and A33R (EEV immunogen) were protected from a lethal poxvirus challenge *(47)*. Both antigens induced greater protection than did either antigen alone, suggesting that for complete protective immunity, both IMF and EEV antigens are required *(38)*.

23.4.1.2 Replication and Virulence of Variola Virus

The orthopoxviruses, like all viruses, depend on host cellular mechanisms for DNA replication, protein synthesis, and viral assembly and release *(38)*. The viral attachment to the host cell is carried out through specific cellular receptors on the outer surface of the virus. Although both IMF and EEV forms are infectious, they differ in the way they attach to and enter the host cells. Whereas the EEV form has a more rapid cellular entry and requires uncoating of the virus in the cellular cytoplasm, the IMF form can enter either through the host-cell outer membrane or within a vacuole formed by the invagination of the plasma membrane releasing its core directly into the cytoplasm *(38)*. Once the core enters the host cytoplasm, there is an immediate transcription of viral enzymes that leads to viral uncoating and the release of DNA into the cytoplasm. Next, DNA replication occurs, followed by translation of both structural and nonstructural polyproteins, resulting in viral assembly first as IMF, which is then wrapped in modified Golgi membranes and transported to the periphery of the cell, with subsequent release of the EEV form of the mature virion *(46)*.

The variola virus represents a collection of many distinct strains, which, although difficult to differentiate antigenically or serologically, is suggested by clinical evidence indicating wide variation in mortality caused by different strains during outbreaks in various parts of the world—mortality rates between 5% and 40% in some regions compared with mortality of only 0.1% to 2% in other areas *(38)*. This distinct difference in virulence led to classification of the variola virus as variola major and variola minor. The genetic differences between the variola major and minor strains have been analyzed by RFLP mapping *(48, 49)*. The observed genetic difference between the variola strains most likely reflects differences in their replication and host-cell assembly, leading to greater or lesser virulence. Thus, a variant of variola virus has been described that produced a unique late polypeptide of different size and endonuclease site *(50)*. This marker was demonstrated to be genetically independent, expressed by 14 of the 48 variola strains examined and correlated with variola major and not variola minor.

23.4.1.3 Immune Evasion Strategies

The observed pathogenesis and virulence among the poxviruses is directly related to the ability of different strains to manipulate the immune response mechanisms of the host in which they replicate, such as the ability to produce many proteins that react with the host at both the cellular and systemic levels *(51, 52)*. Thus, a direct inhibition of the host's cellular immune response by the orthopoxviruses has been demonstrated through the production of soluble receptors for interferon-γ (IFN-γ), which prevented the host-produced IFN-γ from binding to its receptor *(53)*.

The potential of genetic manipulation of the orthopoxviruses, leading to expression of novel proteins and evasion of the host immune responses, introduces the potential to engineer a variola virus that may be used as a biological weapon *(38)*. Thus, recombinant vaccinia viruses that have been engineered to express genes encoding cytokines and chemokines were studied extensively to understand the pathogenesis of the orthopoxviruses *(54)*. The introduction of the interleukin (IL)-4 gene, a human interleukin involved in the type-2 immune response, significantly increased viral virulence by downregulating the cellular, type-1 immune responses. Similarly, IL-4–expressing engineered ectromelia virus was found to overcome genetic resistance to this virus in mice and produced an infection characterized by high mortality similar to the disease seen when genetically sensitive mice have been infected with the wild-type virus *(55)*.

23.4.2 Smallpox: Clinical Manifestations

The morbidity and mortality of smallpox depend on various factors, including the type of the smallpox virus (variola major or variola minor), clinical manifestations, previous smallpox vaccination, age, geography, urban or rural setting, underlying immune status, and, for women, pregnancy *(38)*.

The variola virus gains entry into its human hosts through the oropharynx or respiratory tract. Direct inoculation through the skin can also occur as demonstrated by the practice of variolation. The infectious 50% dose of variola virus is not known. From the time of inoculation with the virus, there is an incubation period of 10 to 14 days *(56)*. During this period, the virus replicates in the respiratory tract and disseminates throughout the body during viremia. Transmission of variola virus from infected patients rarely occurs during the incubation period. However, with the onset

of rash, the period of maximum infectivity begins, then gradually subsides until the rash evolves to the point where all scabs have separated. Because the variola virus is present in the scabs, isolation of patients is necessary until the last scab has become separated *(38)*.

23.4.2.1 Ordinary or Classic Smallpox

As noted above, two distinct types of smallpox have come to be recognized over time: major and minor. The prototypical disease, variola major, or ordinary smallpox, usually results in 30% mortality in unvaccinated patients. However, other clinical forms associated with variola major, such as flat-type and hemorrhagic-type smallpox, have been known for severe mortality *(38)*.

Ordinary smallpox can be classified into three distinct clinical presentations or types: (i) *discrete*, in which individual lesions are separated by normal-appearing skin; (ii) *confluent*, in which lesions on any part of the body coalesce; (iii) *semiconfluent*, in which lesions on the face are confluent but are discrete on the rest of the body. Those patients manifesting discrete lesions tended to have a significantly lower mortality rate than did those with confluent lesions (9% vs. 62% in unvaccinated cases in Rao's clinical series) *(38)*.

Disease manifestations begin acutely, with malaise, fever, rigors, vomiting, headache, and backache, and as many as 15% of patients develop delirium. Two to 3 days after the initial symptoms, an enanthem (rash in the oropharynx) appears concomitantly with a cutaneous rash (exanthem) that covers the face, hands, and the forearms. The exanthema will then spread to the lower extremities and subsequently to the trunk. The lesions will quickly progress from macules to papules to vesicles, and eventually to pustular vesicles. Lesions are more abundant on the extremities and the face, and this centrifugal distribution is an *important diagnostic feature*.

It should be emphasized that in distinct contrast with varicella (chickenpox), lesions on the various segments of the body remain generally synchronous in their stages of development. From 8 to 14 days after the onset of the exanthema, the pustules will scab, leaving depressed depigmented scars upon healing. The major sequela of smallpox is the presence of permanent pockmarks, often leading to an extremely disfigured complexion. Corneal scarring resulting in blindness occurs in 1% to 4% of patients *(38)*.

Smallpox in Children

Smallpox in children is clinically similar to that seen in adults, with several notable exceptions *(38)*. Thus, in infants the overall mortality rate often exceeds 40% *(57)*, and in one report *(58)* it reached 85%. In addition, children have a higher incidence of vomiting, conjunctivitis, seizures, and cough *(59)*. Infections during pregnancy were associated with uterine hemorrhage, premature labor, and fetal demise, although no distinct congenital syndrome has been associated with smallpox infection *in utero (57)*.

23.4.2.2 Hemorrhagic Smallpox

This form of smallpox accounts for about 2% to 3% of all cases. Hemorrhagic smallpox can be subdivided into early and late hemorrhagic smallpox.

Early hemorrhagic smallpox is manifested with a different clinical presentation than is ordinary smallpox and would often kill patients before they exhibited the focal rash *(38)*. Patients develop a toxic clinical picture resembling disseminated intravascular coagulation and in most cases rapidly succumb to the disease. Death occurs in excess of 95% in unvaccinated patients with hemorrhagic smallpox.

In late hemorrhagic smallpox, the focal rash appears but hemorrhages occur between the pustules. In general, hemorrhagic smallpox is more common in adults, and pregnant women would appear to be at greatest risk.

Host factors, such as some form of immune system deficiency, rather than a unique variola strain, are believed to be responsible for the development of hemorrhagic smallpox; however, immunologic data are generally lacking *(60)*.

23.4.2.3 Flat-Type or Malignant Smallpox

The flat-type smallpox accounted for about 6% of all cases in the pre-eradication era and occurred most often in children *(38)*. The disease has a mortality rate of more than 95% in unvaccinated patients. The rash characteristically involves flattened, confluent lesions rather than the characteristic firm pustules observed in ordinary smallpox. As with hemorrhagic smallpox, the flat-type illness has been associated with deficient cellular immune responses to the virus.

23.4.2.4 Modified Smallpox

Modified smallpox for the most part has been associated with persons who had been previously vaccinated but whose immunity had waned with time. According to the WHO definition of modified smallpox, the observed difference is related to the character and development of the focal eruption, with crusting being completed within 10 days. The prodrome may or may not be shortened or lessened in severity, but the secondary fever is typically absent *(57)*. Typically, there are fewer skin lesions, and the lesions tend to be more superficial. In addition, the lesions evolve more

rapidly and do not show the typical uniformity observed in ordinary smallpox. Diagnosis of modified smallpox is more difficult, and patients are still contagious, although often ambulatory *(38)*.

23.4.2.5 Variola Sine Eruptione

Variola sine eruptione occurs in previously vaccinated for smallpox after exposure to smallpox and is characterized by sudden onset of fever, headache, and backache, but *no rash followed the prodrome*. The illness usually is short lived and resolves in 1 to 2 days. However, patients can still transmit the virus to others for a short period of time *(38)*.

23.4.2.6 Variola Minor

The second form of smallpox, variola minor, is distinguished by milder systemic toxicity, a lesser degree of pox lesions, and a mortality rate of 1% in unvaccinated persons. Although the viral exanthema is the most prominent feature of both forms of smallpox, when patients with variola major and variola minor were matched for the same extent of exanthema, mortality rates differed substantially. Patients with variola minor illness can be confused with those presenting with modified smallpox *(38)*.

23.4.3 Treatment of Smallpox

In the pre-eradication era and through the last human case of smallpox infection in 1978, the standard treatment consisted of supportive measures such as hydration, fever and pain control, and meticulous care for the skin to prevent bacterial superinfection *(38)*.

Although drug research has found a new momentum as the threat of biological weapons attack has been fully appreciated, very few drugs against orthopoxviruses have advanced to the stage of human trials and, if they did, the results were disappointing *(61, 62)*. Among the first antiviral drugs tested for anti-orthopoxvirus activity were cytosine arabinoside and adenine arabinoside, and the thiosemicarbazones (methisazone, M&B 7714). The latter showed excellent activity in mouse models of vaccinia and variola *(61, 62)*. However, randomized, placebo-controlled human trials for treating smallpox infection yielded disappointing results *(63,64)*. In addition, the adverse side effects (gastrointestinal, nausea, vomiting) were severe, and the expense of the thiosemicarbazones is prohibitive *(61, 62)*.

Other antiviral compounds that have shown *in vitro* activity against orthopoxviruses included inosine monophosphate (IMP), dehydrogenase inhibitors, *S*-adenosylhomocysteine hydrolase inhibitors, orotidine monophosphate decarboxylase inhibitors, cytosine triphosphate synthetase inhibitors, thymidylate synthetase inhibitors, nucleoside analogues, thiosemicarbazones, acyclic nucleoside phosphonates, and carbocyclic imidodisulfamide analogues *(61, 62, 65–68)*. Most of the *in vitro* activity has been determined against orthopoxviruses other than variola, but several classes of the available antiviral drugs were found to be specifically active against the variola virus in addition to other orthopoxviruses *(66–68)*.

Of the current commercially available drugs, HPMPC (cidofovir), *bis*-POM PMEA (adefovir dipivoxil), and ribavirin have been shown to have an IC_{59} in cell culture assays well below the levels associated with cytotoxicity *(61,62,65)*. Both cidofovir and adefovir dipivoxil have been associated with severe nephrotoxicity.

Whereas no human data exist for cidofovir for treating smallpox, there were case reports documenting success in its use against other poxvirus infections. In addition, topical and parenteral cidofovir have been used to effectively treat patients with severe immunodeficiency disorders (AIDS and Wiskott-Aldrich syndrome) and disfiguring molluscum contagiosum *(69, 70)*.

An orally bioavailable anti-poxvirus compound (ST-246) was found to inhibit extracellular virus formation and protected mice from lethal orthopoxvirus challenge. ST-246 has been shown to be highly active against a number of orthopoxviruses both *in vitro* and in animal models. These results, coupled with its lack of toxicity, make ST-246 a very promising candidate for further development to prevent or treat smallpox infection in humans *(71)*.

23.4.4 Smallpox Vaccines

Several different vaccinia strains have been used in the production of vaccines against smallpox *(38)*. Currently in the United States, the Dryvax vaccine (Wyeth) is the only licensed vaccinia-based vaccine. It consists of a lyophilized (freeze-dried) preparation of live vaccinia virus obtained from New York City calf lymph, which is derived from a seed virus of the New York City Board of Health (NYCBH) strain. Other strains used in preparing smallpox vaccines also include the New York City chorioallantoic membrane (also derived from the NYCBH strain), EM-63 (former Soviet Union), the temple of heaven strain (China), and the Lister or Elestree strain (United Kingdom). Whereas all strains seemed to be equally effective in preventing smallpox, a difference existed in the adverse reactions *(38)*.

A vaccinia strain referred to as *modified vaccinia Ankara (MVA)* was successfully used in Europe throughout the 1970s. The attenuated vaccinia virus in MVA appeared to have less virulence than did the other vaccinia strains, especially with regard to postvaccination encephalitis *(38)*.

23.5 Yersinia pestis: Plague

Plague has been associated with three pandemics—in the 6th, 14th, and 20th centuries. During World War II, the Japanese Army apparently used plague as a biological weapon in China.

The causative agent of plague is *Yersinia pestis*, a Gram-negative, nonacid-fast, nonmotile, nonsporulating, nonlactose-fermenting, bipolar coccobacillus. The genus *Yersinia* (family Enterobacteriaceae) consists of 11 species, of which three are human pathogens (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*). In common with other enteric bacteria, *Y. pestis* has typical cell wall and whole-cell lipid composition and an enterobacterial antigen *(72)*. A facultative intracellular pathogen, *Y. pestis*, is now thought to maintain intracellular residence only during the early stages of infection, with extracellular growth being predominant at later stages.

All three human pathogens (*Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*) have a nearly identical siderophore-dependent iron transport system. The majority of *Y. pestis* strains, regardless of biotype or origin, contain three plasmids termed pPCP1 (pesticin, coagulase, plasminogen activator), pCD1 (calcium dependence), and pMT1 (murin toxin). A low-calcium response stimulon (LCRS) is encoded on pCD1 and includes regulatory genes controlling the expression of secreted virulence proteins and a dedicated multiprotein secretory system. The LCRS region is highly conserved, and the LCRS plasmids are necessary for virulence in all three human pathogenic *Yersinia* species *(72)*.

The naturally occurring disease is transmitted to humans from rodents by fleas. The most common form of plague is the *bubonic plague* (about 90%). Secondary *septicemic plague* occurs in about one fifth of patients and secondary *pneumonic plague* in about 10%. If *Y. pestis* were used as a biological weapon, the primary manifestation would be epidemic pneumonia. The transmission of pneumonic plague from person to person is extremely high *(7, 72)*.

Among the numerous animal models used over the years, those that exhibit the closest relationship to pathology in humans include the mouse and non-human primate (NHP) models *(73–77)*. The pathology of plague infection in humans and NHPs is strikingly similar.

23.5.1 Epidemiology of Y. pestis

Plague is a zoonotic disease primarily affecting rodents; humans play no role in the long-term survival of *Y. pestis*. Transmission between rodents is accomplished by their associated fleas. Whereas infection can occur by direct contact or ingestion, these routes normally do not play a role in the maintenance of *Y. pestis* in an animal reservoir. Fleas will acquire the pathogen from an infected blood meal—the oriental rat flea (*Xenopsylla cheopis*) is the classic vector for plague. Another flea, *Xenopsylla astia*, is also a known vector of *Y. pestis (72)*. Currently, only a small number (around 31) of flea species were proven as vectors of plague. The cat flea (*Ctenocephalides felis*) and the so-called human flea (*Pulex irritans*) were found to be very poor vectors.

Y. pestis has also been isolated from lice and ticks. However, there is no evidence yet for transmission of plague by ticks to mammals.

Through the start of the third pandemic, transmission from urban rodents (especially rats) was most common. Currently, most human plague cases in the world and all cases in the United States are classified as sylvatic plague, contracted from rural wild animals, such as squirrels, chipmunks, marmots, voles, gerbils, mice, and rabbits *(72)*. In the United States, transmission to humans occurs primarily via the bites of fleas from infected rodents.

It is accepted that the pathogen spreads from the site of the flea bite to the regional lymph nodes and grows to high numbers, causing the formation of a *bubo* (swollen lymph node). Then, the infection spreads into the bloodstream, where the bacilli are preferentially removed in the spleen and liver but also colonize other internal organs.

23.5.2 Bubonic Plague

Human epidemics generally start as bubonic plague, which is the classic form of the disease. When a bubonic plague victim develops secondary pneumonic plague, the potential for respiratory droplets spreads, and a primary pneumonic plague epidemic occurs. However, this type of epidemic is currently uncommon because of the advent of effective antibiotics and modern public health measures *(72)*.

Patients with bubonic plague usually develop symptoms of fever, headache, chills, and swollen, extremely tender lymph nodes (buboes) within 2 to 6 days after contact with the pathogen. In addition, gastrointestinal complaints, such as nausea, vomiting, and diarrhea, are common. Depending on the duration of the infection, symptoms of disseminated intravascular coagulation (DIC) can also develop. Skin lesions infrequently develop at the initial site of the infection. Buboes are typically found in the inguinal and femoral regions but can also occur in other nodes *(78)*. Bacteremia or secondary plague septicemia is frequently seen in patients with bubonic plague *(72)*.

Intracellular *Y. pestis* survive by an undefined mechanism, but most eventually escape their intracellular captivity within the lymph node *(77)*. Within hours of the infection, extracellular bacteria in the lymph nodes express a range of anti-

host proteins, including the proteinaceous Fraction One (F1) capsule *(72)*.

23.5.3 Septicemic Plague

Primary septicemic plague is generally defined as occurring in patients with positive blood cultures but no palpable lymphadenopathy. Clinically, septicemic plague resembles septicemia caused by other Gram-negative bacteria, during which patients present with chills, headache, malaise, and gastrointestinal disturbances. Compared with patients with bubonic plague, patients with septicemic disease have a higher incidence of abdominal pain. The mortality rate of patients with septicemic plague is fairly high, ranging from 30% to 50%, probably because the antibiotics generally used to treat undifferentiated sepsis are not effective against *Y. pestis (72, 79)*.

During the development of septicemic plague, it is not clear if the invading bacteria actually avoid the regional lymph nodes or, if infection of the nodes does occur, the invading bacteria fail to induce gross pathologic or physiologic changes *(80)*.

23.5.4 Pneumonic Plague

Pneumonic plague is a rare form of the disease with a very high mortality rate *(72)*. It is spread through respiratory droplets by close contact (2 to 5 ft) with an infected person. It progresses rapidly from a febrile flu-like illness to an overwhelming pneumonia, manifested by coughing and the production of bloody sputum. The incubation period of pneumonic plague is between 1 and 3 days. In the United States, the vast majority of cases were contracted from infected cats; during the period 1970–1993, 12% of the U.S. plague patients developed pneumonia secondary to either the bubonic or septicemic form of plague *(81)*.

Clinical disease after exposure to "intentionally" aerosolized plague is markedly different *(77)*. Primary pneumonic plague occurs after inhaling plague bacilli dispersed in a size that can reach the deep lungs (1 to 10 μm in diameter). Deposited bacilli may be unencapsulated and phenotypically negative for the virulence-associated *Yersinia* outer proteins (Yops) or, if transmitted from an infected person, may be expressing both. The effect of expressing or not expressing capsule and/or Yop proteins in the lungs at the time of infection is still not clear *(77)*.

An asymptomatic infection in some humans and nonhuman primates (NHPs) presumably exposed to aerosolized plague is *pharyngeal plague (82, 83)*. Pharyngeal carriage is thought to eventually resolve asymptomatically or progress after an extended time to fulminate into plague pneumonia *(77)*.

23.5.5 Treatment of Plague

All patients suspected of having bubonic plague should be placed in isolation until 2 days after starting antibiotic treatment to prevent the potential spread of the disease should the patient develop secondary plague pneumonia *(72)*.

Numerous antibiotics have been used to treat *Y. pestis* infections or as prophylaxis. Streptomycin has for many years remained the drug of choice. It is given intramuscularly to adults at a daily dose of 2.0 g, twice daily (at 12-hour intervals); the dose in children is 30 mg/kg, 2 to 3 times daily. Because streptomycin is bacteriolytic, it should be administered with care to prevent the development of endotoxic shock. Because of its toxicity, patients are not usually maintained on streptomycin for the full 10-day treatment regimen but are gradually switched to one of the other antibiotics, usually tetracycline.

The tetracyclines are commonly used for prophylactic therapy. In adults, tetracycline is administered at an oral daily dose of 2.0 g (4 times daily at 6-hour intervals); the oral daily dose for children ($\geq$ 9 years) is 25 to 50 mg/kg (4 times daily at 6-hour intervals). Chloramphenicol is typically used to treat plague meningitis.

Whereas *Y. pestis* is susceptible to penicillin *in vitro*, the antibiotic is considered to be ineffective against plague. Antibiotic-resistant strains of *Y. pestis* are rare and are not increasing in frequency *(72)*.

23.5.5.1 Killed Whole-Cell Plague Vaccines

Historically, whole-cell plague vaccines are effective in preventing bubonic plague in humans. The basis for their inability to protect against pneumonic plague is not well understood; it may be caused by alteration in the quality or quantity of the F1 antigen and the limited presence of other important proteins. A major criticism of the whole-cell plague vaccines is the predicted duration of "immunity" and the inability to induce a mucosal immune response *(77)*.

The Haffkine Vaccines. First developed in India in the 1890s by W. M. W. Haffkine *(84, 85)*, the original vaccine was a whole-cell killed vaccine made from stationary-phase broth cultures of the virulent Indian strain of *Y. pestis* 195/P. An improved Haffkine vaccine was subsequently produced using log-phase broth cultures grown at 37°C. A retrospective comparison of the old and new Haffkine vaccines revealed that the latter contained 4 times the amount of F1 antigen, which had been directly linked to its greater efficacy. Although the Haffkine vaccine was reported to be relatively effective, its drawbacks included severe side effects, a high number of vaccine failures, and contamination of some of the vaccine preparations. It was ultimately abandoned in favor

of the development of the new live-attenuated plague vaccines *(77)*. In addition, there has been no evidence that the Haffkine-type vaccines were effective against aerosol challenge by *Y. pestis*.

The "Army" Vaccine. In 1941, the U.S. National Research Council recommended the use of a new "Army" whole-cell killed plague vaccine for deploying U.S. troops. The decision to use this vaccine was based on its less severe vaccine reactions in humans compared with that of the Haffkine vaccine, and it was credited for reducing bubonic plague morbidity and mortality of U.S. soldiers during World War II *(77)*. Later, with minor modifications, the Army vaccine became the Plague Vaccine USP. As with the Haffkine vaccine, there is no evidence that the Army vaccine protected against pneumonic plague.

The Cutter/Greer Vaccines. The first U.S. licensed vaccine was manufactured by Cutter Co. and was an adaptation of the Army product *(77)*. The Cutter vaccine underwent several modifications, with a final formulation developed in 1967 and its subsequent manufacture by Greer Laboratories. The Greer vaccine was prepared from fully virulent *Y. pestis* 195/P. The principal protective antigen in the Cutter/Greer vaccines is the capsular antigen F1 because the induction of antibodies to F1 was considered essential for the development of a protective immune response.

23.5.5.2 Live-Attenuated Plague Vaccines

The first live-attenuated plague vaccines were tested for efficacy in large-scale trials in the 1930s using the Tjiwidej and EV strains. Of the two vaccines, the EV-type vaccine was more extensively used and reported to be more effective *(86)*. Whereas the Tjiwidej strain was reported negative for the production of V antigen (VW-), the EV-type vaccine did produce V antigen *(77)*. Trials in NHPs indicated that the EV-type vaccines exhibited significant protection from bubonic plague after parenteral challenge. There is also evidence that the live-attenuated plague vaccines were effective in reducing the incidence of bubonic plague in humans *(87)*.

A potential problem with the EV-type vaccines has been the maintenance of seed cultures, as the EV-76 strain has undergone several changes after its original isolation, and these cultures are known to change phenotypes *(88)*. On average, only about 30% of a delivered dose of EV-type vaccines is viable. Currently, there is no methodology to control for this fluctuation in viability. This aspect of the EV-type vaccine has significant implications for vaccinating elderly, young, and immunocompromised populations *(77)*.

Very little is known concerning protection from pneumonic plague with live-attenuated plague vaccines. There is one published report of EV-76–dependent protection in rhesus monkeys intratracheally challenged *(89)*. Resistance in vaccinated monkeys was related to circulating levels of anti-F1 antibody.

Serious side effects caused by live-attenuated plague vaccines were first reported in Africa (Senegal) then in the former Soviet Union and included fever, lymphadenopathy, malaise, headache, and sloughing of skin at the inoculation side *(90)*. These and other reports of adverse side effects raised serious questions about the acceptability of the EV-type live-attenuated plague vaccines, especially for mass vaccination. In addition, the induction of long-term immunity may also be a problem with the EV-type vaccines—as determined from intradermal inoculation of pestin, immunity may wane in 60% of vaccinees between 6 months and 1 year *(91)*. It was noted that in general, prior vaccination with a killed plague vaccine had an ameliorating effect on subjects given the live vaccine *(77)*.

23.5.5.3 Subunit Plague Vaccines

The subunit vaccines are essentially acellular preparations that contain one or a number of bacterial antigens, proteins, and/or carbohydrates, formulated with a characterized adjuvant *(77)*. Because the basis for selecting purified plague antigens is their immunogenicity in the host during infection, it should be noted that not all plague immunogens by any means induce a protective response in the host.

Capsular F1 Antigen. Y. pestis has long been known to produce a highly immunogenic, proteinaceous capsular antigen F1 that is actively synthesized both *in vitro* and *in vivo (92)*. The F1 antigen, regardless of the source and whether cell-associated or cell-free, provides a high degree of protection against either lethal parenteral (SC) or aerosolized plague infection. However, because the capsular F1 antigen may not be the only determinant of virulence that induces protective immunity in the host *(93)*, additional protective components may also be included in the vaccine preparation, giving an added advantage to the protected host.

Lipopolysaccharide (LPS). In addition to large amounts of F1 capsule, Plague Vaccine UPS also contains very high levels of LPS, which may be a contributing factor to the reactogenicity of this product observed in many recipients of the vaccine *(94)*. Like most Gram-negative bacteria, the *Y. pestis* LPS contains 2-keto-3-deoxyoctulosonic acid but is lacking a true *O*-side-chain antigen, probably as the result of mutations in existing genes required for the biosynthesis of the *O*-antigen. Even though the immunomodulatory effects of LPS are unquestionable, in light of experimental evidence that purified LPS failed to protect mice *(95)*, the contribution of *Y. pestis* LPS in the development of a protective immune response in the host remains tenuous *(77)*.

pH 6.0 Antigen. The pH 6.0 antigen is a fibrillar-like protein polymer. Like the F1 capsule, the pH 6.0 antigen

is a protein structure that appears across the surface of the pathogen as filamentous strands only at 37°C *(96)*. However, unlike the F1 antigen, a low pH is required for the expression of pH 6.0 antigen. This phenomenon is highly suggestive for the selective expression of the antigen in acidified host cell compartments such as the phagolysosome of a macrophage *(97)*. Nonetheless, purified preparations of recombinant pH 6.0 antigen protein failed to protect inoculated mice against parenteral plague challenge—although the protein induced very high levels of circulating antibody *(77)*.

Plasminogen Activator (Pla). The plasminogen activator is a protein encoded by the small virulence plasmid unique to *Y. pestis (77)*. It possesses an enzymatic activity that leads to clot lysis by converting plasminogen to the plasmin protease *(98)*. The bacterial plasminogen activator activity is not unique to *Y. pestis* and is associated with the virulence of many bacterial species *(99)*. It has been hypothesized that Pla has a dual enzymatic function: as a dissemination factor in the mammal, and as a clotting factor in the flea. However, as with the pH 6.0 antigen, preliminary studies evaluating the efficacy of recombinant Pla in mice yielded inconclusive results *(77)*.

V Antigen. The virulence determinant known as the V antigen present in *Yersinia* species is required for full virulence of these bacteria *(100)*. This polypeptide is encoded by the medium-molecular-weight virulence plasmid pLCR and is temperature-regulated (expressed only at 37°C). It is induced *in vitro* only in the absence of calcium cations, hence the term *low calcium response*. It is thought that the function of the V antigen is regulatory and associated with secretions of other virulence proteins *(101, 102)*. In addition, there has been experimental evidence suggesting host immunomodulatory effects *(103, 104)*.

The use of the V antigen as a protective immunogen was documented several decades ago *(105)*. In a recent study, purified recombinant V antigen demonstrated excellent efficacy against high-level parenteral and aerosol challenges *(106)*. These and other results have strongly suggested using the V antigen as at least one of the components in a new acellular vaccine *(77)*.

Other pLCR-Encoded Proteins. Additional antigenic proteins identified on pLCR that could be used as subunit vaccine candidates include a group of effector proteins (known as the *Yersinia* outer proteins, or Yops) that are translocated intracellularly by a type-III secretion apparatus *(77)*. Various biochemical functions have been defined for these proteins, including apoptosis (YopJ), cytolysis (YopB/D), dephosphorylation of host cell proteins (YopH), serine/threonine kinase activity (YpkA), actin microfilament disruption (YopE), and inhibition of platelet aggregation (YopM). Studies have shown that at least some Yops are immunogenic, as antibodies to these proteins have been detected in human convalescent sera *(107)*.

Multiple Subunit Vaccines. The preponderance of experimental evidence collected to date strongly suggests that a rational approach for new cell-free plague vaccine would consist of both the F1 capsule and V antigens *(77)*. In this regard, a unique recombinant hybrid protein, consisting of a gene fusion between the F1 structural gene *caf1* and *lcrV* encoding V antigen, has been generated *(108)*. The gene product was then expressed in *E. coli* to reasonably high levels and purified by standard chromatographic techniques. In a single-dose vaccination study, the F1-V fusion protein has shown a synergistic effect as demonstrated by its ability to protect against very high doses of virulent plague organisms *(109)*.

One great advantage of the F1-V fusion protein is its easy manufacturing, as the components of an F1 plus V mixture would be considered as separate products.

Another approach to a multiple subunit vaccine would be to use a “complex” of antigens *(77)*. In the case of *Yersinia*, the most likely vaccine candidate would be the YopB/D cytolytic porin. This bipartite complex will attach to the target host cell to form a “portal of entry” for the pathogen’s effector proteins *(110)*. It is hypothesized that when the YopB/D complex is used, an improved and potentially synergistic immune response may be developed, with the caveat of potential toxicity to the host *(77)*.

23.6 Brucellosis

Brucellosis is a chronic, systemic, febrile, granulomatous infection caused by at least four different *Brucella* species *(111, 112)*. The latter are Gram-negative, aerobic, nonmotile, nonspore-forming intracellular coccobacilli. As intracellular bacteria of mononuclear phagocytes, the *Brucella* species successfully evade many host immune responses and resist easy eradication by antimicrobial agents. Because of its great infectivity, ability to incapacitate infected individuals, and the persistent nature of human disease, brucellosis has long been considered a prime biowarfare threat. Both the United States and the former Soviet Union weaponized *Brucella* in the 1940s *(111)*.

The traditional classification of *Brucella* species is largely based on their preferred hosts. There are six classic pathogens—*Brucella melitensis*, *B. abortus*, *B. suis*, *B. canis*, *B. ovis*, and *B. neotomae*—of which the first four are recognized human zoonoses. The presence of rough or smooth lipopolysaccharide (LPS) correlates with the virulence of the disease in humans. New *Brucella* species, provisionally called *B. pinnipediae* and *B. cetaceae*, were recently isolated from marine mammals and found to be pathogenic to humans *(111, 112)*. *Brucella ovis* and *B. neotomae* are not known to cause human disease.

Taxonomically, *Brucella* is a monospecific genus that should be termed *B. melitensis*, and all other species are subtypes with an interspecies homology above 87%. The phenotypic difference and host preference may be attributed to various proteomes, as exemplified by specific outer-membrane markers *(112, 113)*.

The complete sequencing of the *B. melitensis* genome was determined in 2002 *(114)*. The complete genome sequencing of two other *Brucella* species, *B. abortus (115)* and *B. suis (116)*, have also been accomplished. The genome of *Brucella melitensis* reveals the presence of two circular replicons of 1.1 and 2.2 Mb, respectively, with a 57% CG content and no plasmids. The *B. abortus* biovars 1 and 4 and *B. suis* biotype 1 were found to be remarkably similar to *B. melitensis*. In contrast, *B. suis* biotypes 2 and 4 are composed of two replicons of 1.35 and 1.85 Mb, respectively, and *B. suis* biotype 3 is composed of a single circular replicon of 3.3 Mb *(112)*.

23.6.1 Pathogenesis of Brucella Species

The *Brucella* species do not produce exotoxins and do not naturally harbor plasmids or phages. Their most remarkable virulence factor is the outer membrane LPS *(111)*. At least two features contribute to the ability of LPS to enhance bacterial survival in the host, namely (i) the endotoxic activity (ability to trigger a systemic inflammatory response) of LPS is much less than that of typical enteric Gram-negative organisms, so innate immune responses are poorly activated by encounters with the bacterium; and (ii) smooth strains of *Brucella*, which have long chains of *O*-polysaccharide (OPS) on their LPS, fix small amount of serum complement to their surface but are resistant to complement-mediated lysis *(117)*. Moreover, smooth strains have a reduced ability to induce cytokine responses from the monocytes, most likely because of steric interference by their surface OPS with the binding of the lipid A endotoxic component of LPS to mononuclear phagocyte surface receptors *(111)*. Furthermore, smooth, virulent brucellae that have been coated with complement are readily phagocytosed by mononuclear phagocytes. Inside these cells, brucellae foster phagosomal acidification, inhibit the fusion of phagosomes with lysosomes, remain in the phagosomes, and replicate to an enormous number inside their host cells *(111)*.

Brucellae are resistant to damage from polymorphonuclear cells due to the suppression of the myeloperoxidase–hydrogen peroxide–halide system and copper-zinc superoxide dismutase, and the production of inhibitors of adenylate monophosphate and guanyl monophosphate *(112)*. Impaired activity of the natural killer cells and impaired macrophage generation of reactive oxygen intermediates and interferon regulatory factors have also been documented *(118–120)*. Interferon-γ has a central role in the pathogenesis of brucellosis *(121, 122)* by activating macrophages, producing reactive oxygen species and nitrogen intermediates; by inducing apoptosis, enhancing cell differentiation and cytokine production; by converting immunoglobulin G to immunoglobulin G2a; and by increasing the expression of antigen-presenting molecules *(112)*.

After the lysis of infected cells, the bacteria are ingested by other mononuclear phagocytes, and the cycle of bacterial proliferation will continue. However, with the development of an effective host response, the bacterial proliferation is controlled and brucellae are gradually eliminated. Nevertheless, the bacteria may persist in their host cells for months or even years and recommence replication if the activity of immunologic control mechanisms declines *(111)*. In addition to mononuclear phagocytes, placental trophoblasts are also highly susceptible to infection with brucellae and support rampant intracellular proliferation of the bacteria. In this location, brucellae associate with the rough endoplasmic reticulum *(123)*, where they may have access to more nutrients than are available in the macrophage phagosome.

23.6.2 Clinical Manifestations of Brucellosis

In humans, *B. melitensis* is the most virulent species, followed by *B. abortus* and *B. suis*. *Brucella canis* and *B. maris* (isolated from marine mammals) appear to be approximately as virulent as *B. abortus*.

Transmission of brucellosis to humans occurs through the consumption of infected, unpasteurized animal milk products (raw milk, soft cheese, butter, and ice cream), through direct contact with infected animal parts (such as placenta by inoculation through ruptures of skin and mucous membranes), and through the inhalation of infected aerosolized particles *(112)*. Airborne transmission of brucellosis has been studied in the context of using brucellae as a biological weapon *(111)*.

After brucellae encounter a susceptible host, the bacteria enter across the mucous membranes and are ingested by mononuclear phagocytes, then travel to local lymph nodes, and disseminate via the thoracic duct and blood throughout the mononuclear phagocyte system *(111)*. At the time of dissemination, humans typically present with fever, chills, and malaise. In addition, neuropsychiatric abnormalities, including depression and inability to concentrate on task performance, are common *(124)*.

Focal disease may occur in almost any organ but more often tend to develop in sites where blood supply is particularly rich. Osteoarticular disease, which is universally the most common complication of brucellosis, is manifested in three distinct forms: peripheral arthritis, sacroiliitis, and spondylitis. Approximately one third of patients will have

disease in vertebrae or in one or more joints (knees, hips, ankles, wrists, and especially the sacroiliacs). *Brucella* vertebral osteomyelitis, which may be clinically indistinguishable from tuberculosis, tends to develop in older patients. Spondylitis remains very difficult to treat and often seems to result in residual damage *(125)*.

The reproductive system is the second most common site of focal brucellosis. Epididymitis or epididymoorchitis occur in about 2% to 10% of male patients. Brucellosis in pregnancy poses a serious risk of spontaneous abortion *(126)*.

Endocarditis and CNS disease occur in 2% to 5% of patients; meningitis, encephalitis, meningoencephalitis, meningovascular disease, brain abscesses, and demyelinating syndromes have all been reported *(127)*. However, endocarditis remains the principal cause of morbidity in brucellosis and the major cause of death. It usually involves the aortic valve and typically requires immediate surgical valve replacement *(111, 112)*.

The blood count is often characterized by mild leukopenia and relative lymphocytosis, along with mild anemia and thrombocytopenia. The thrombocytopenia may be severe *(111)*.

Relapses, which are rare (about 10% of cases), usually occur in the first year after infection and are often milder in severity than is the initial disease.

In contrast with humans, livestock do not show systemic signs of disease during the dissemination phase. Brucellae have also been found in milk macrophages, leading to infection in young animals and providing a source of human infection.

Diagnosis of brucellosis is usually made serologically based on the detection of antibody directed against OPS.

23.6.3 Treatment of Brucellosis

Treatment of human brucellosis should involve antibiotics that can penetrate macrophages and can act in the acidic intracellular environment. There is a general need for antibiotic combination treatment because all monotherapies are characterized by unacceptably high relapse rates *(111, 112)*. In addition, the general discrepancy between *in vitro* activity and *in vivo* observations will preclude the study of resistance patterns of brucellae or *in vitro* evaluation of the efficacy of individual antibiotics.

In 1986, WHO issued guidelines for the treatment of human brucellosis. The guidelines discussed two regimens, both using doxycycline (100 mg, twice daily) for a period of 6 weeks, in combination with either streptomycin (15 mg/kg, intramuscularly) for 2 to 3 weeks or rifampin (600 to 1,200 mg daily) for 6 weeks *(112)*.

Alternative drug combinations have also been used, including aminoglycosides such as gentamicin (5.0 mg/kg daily in 3 divided intravenous doses for 5 to 7 days) and netilmicin. The trimethoprim-sulfamethoxazole combination (960 mg, twice daily for 6 weeks) is usually used in triple regimens. Various combinations that incorporate ciprofloxacin (500 mg, twice daily for 6 weeks) and ofloxacin (400 mg, twice daily for 6 weeks) have been tried clinically and found to have similar efficacy as those of the classic regimens *(112)*.

In various combinations, rifampin is the drug of choice in the treatment of brucellosis in pregnancy. Brucellosis in children has been treated with a combination based on rifampin and trimethoprim-sulfamethoxazole *(112)*.

23.6.3.1 Human Brucellosis Vaccines

It is likely that a human vaccine against brucellosis will elicit both humoral and cellular immune responses. However, although several vaccines have been tested in the past, none was completely satisfactory *(128)*. Vaccines from *B. abortus* strain 19 were used in the former Soviet Union, and strains of *B. abortus* 104M have been used in China. A phenol-insoluble peptidoglycan fraction of *B. melitensis* strain M15 was used in France *(111, 112)*.

Theoretical vaccine targets for use in future vaccine development may include the *rfbK* mutations of *B. melitensis*, the outer-membrane protein 25, and the cytoplasmic protein BP26 *(129)*.

23.7 Coxiella burnetii: Q Fever

Q fever was first discovered in Australia as an occupational disease and was found in the United States just before the outbreak of World War II. Currently, this zoonotic disease caused by *Coxiella burnetii* has nearly worldwide distribution *(130, 131)*. Cattle, sheep, and goats are the primary reservoirs of *C. burnetii*. Organisms are excreted in the milk, urine, and feces of infected animals. However, *C. burnetii* infection has been noted in a wide variety of other animals, including other species of livestock and domesticated pets, as well as arthropods. *C. burnetii* does not usually cause clinical disease in these animals, although abortion in goats and sheep has been linked to *C. burnetii* infection. Humans are the only hosts identified that normally would experience an illness as a result of infection (http://cdc.gov/ncidod/dvrd/qfever). *C. burnetii* is resistant to heat, drying, and many common disinfectants, which enables the bacteria to survive in the environment for long periods.

Infections in humans usually occur from inhaling these organisms from air that contains airborne barnyard dust

contaminated with dried placental material, birth fluids, and excreta of infected herd animals. Ingestion of contaminated milk, followed by regurgitation and inspiration of the contaminated food, is a less common mode of transmission. Other modes of transmission to humans, including tick bites and human-to-human transmission, are rare.

Humans are often very susceptible to the disease, and very few organisms may be required to cause infection. Because of its infectivity, *C. burnetii* could be developed for use in biological warfare and is considered a potential terrorist threat.

Coxiella burnetii is an obligated intracellular organism that grows as a small coccobacillus, approximately 0.8 to 1.0 μm in length by 0.3 to 0.5 μm in width, and may occur either singly or in short chains. It is classified in the family Rickettsiaceae but is not included in the genus, and therefore is not a true rickettsia. Moreover, it was not found to be closely related to any other bacterial species when comparative 16s ribosomal ribonucleic acid analysis was performed. Thus, the genus *Coxiella* has only one species. Its closest relative, according to 16s ribosomal RNA analysis, is *Legionella (132,133)*, even though *Legionella* has different growth characteristics—the ability to survive and multiply extracellularly and causing a different clinical syndrome *(131)*. In contrast, *C. burnetii* cannot have sustained growth and replication outside a host cell.

C. burnetii replicates only within the phagolysosomal vacuoles of animal cells, primarily macrophages. The replication occurs by binary fission.

The most important biological feature of the organism is the existence of small, compacted cell types within mature populations growing in animal hosts. These forms, called small-cell variants (SCVs), are absolutely distinct from the large-cell variants (LCVs) in the population. The latter are likely the metabolizing stage in what is obviously a developmental life cycle in *C. burnetii (130)*. It is thought that the SCVs of the developmental cycle of the organism are mainly linked to the unusual resistance characteristics of *C. burnetii*, as well as its long-term durability within different environments.

The virulent *C. burnetii*, which is usually linked to natural infection and a smooth LPS, is designated as phase I. This phase is resistant to complement and is a potent immunogen. Serial passage of *C. burnetii* in eggs eventually results in the bacterium's conversion to phase II, which has a rough LPS and is much less virulent than phase I. Phase II is sensitive to complement and is a poor immunogen. The conversion of phase I to phase II is irreversible and is the result of a mutation caused by a chromosomal deletion *(131)*.

C. burnetii also contains several plasmids, and dissimilar plasmid types may be associated with different manifestations of disease *(134)*. In addition, the cell wall of a phase I *C. burnetii* organism contains, in association with the LPS, an immunomodulatory complex *(135)* that produces toxic reactions in mice (e.g., hepatomegaly, splenomegaly, liver necrosis) and lymphocyte hyporesponsiveness *in vitro (131)*.

23.7.1 Q Fever: Clinical Manifestations

C. burnetii is extremely infectious; under experimental conditions, a *single organism* is capable of producing infection and disease in humans *(136)*. Human infection is usually the results of inhalation of infected aerosols. The incubation period varies from 10 to 40 days, with its duration being inversely correlated with the magnitude of the inoculum—a higher inoculum also increases the severity of the disease *(131)*.

Q fever in humans may be manifested as asymptomatic seroconversion, acute illness, or chronic disease. Infection with *C. burnetii* has been reported to persist in humans (as it does in animals) in an asymptomatic state. Although possible, infection with Q fever may adversely affect the outcome of pregnancy.

Q fever is usually diagnosed by serologic testing because culture of *C. burnetii* is potentially dangerous to laboratory personnel and requires animal inoculation or cell culture. A number of serologic methods have been used, including complement fixation, indirect fluorescent antibody, macroagglutination and microagglutination, and the enzyme-linked immunosorbent assay (ELISA). Of the methods currently used for serologic testing of Q fever, ELISA is the most sensitive (80% to 84% in early convalescence, and 100% in intermediate and late convalescence) *(137)* and easiest to perform. In general, antibodies to the rough phase II organism are identified earlier in the illness, during the first few months after infection, followed by a decline in antibody to phase II organisms and a rise in antibody to the smooth, virulent phase I organism *(131)*.

23.7.1.1 Acute Q Fever

There is no characteristic illness for acute Q fever, and manifestations may vary considerably among locations where the disease is acquired *(131)*.

When symptomatic, the onset of Q fever may be abrupt or insidious, with fever, chills (including frank rigors), and headache (usually severe, throbbing, and frontal or retroorbital in location) being the most common. Diaphoresis, malaise, fatigue, and anorexia are also very common. Relatively infrequent symptoms include sore throat, gastrointestinal upset, and neck stiffness; the last symptom may be severe enough to require a lumbar puncture to exclude bacterial meningitis *(131)*.

A common clinical manifestation of Q fever is pneumonia *(138)*. Atypical pneumonia is most frequent, and asymptomatic patients can also exhibit radiologic changes (usually nonspecific, but also rounded opacities and hilar adenopathy).

Neurologic complications were observed in up to 23% of acute cases, including encephalopathic symptoms, hallucinations (visual and auditory), expressive dysplasia, hemifacial pain resembling trigeminal neuralgia, diplopia, and dysarthria. Other symptoms involving the CNS, such as encephalitis, encephalomyelitis, optic neuritis, or myelopathy, may also occur, particularly late in acute illness *(131)*.

23.7.1.2 Chronic Q Fever

Chronic infection with *C. burnetii* is usually rare but, unlike acute illness, is often fatal *(130)*. Chronic disease occurs almost exclusively in patients with prior coronary illness or in immunocompromised patients with AIDS or cancer or transplant recipients.

Chronic Q fever is manifested mainly by infective endocarditis, which also is the most severe complication of Q fever. Other syndromes, such as chronic hepatitis and infection of surgical lesions, have also been observed *(130, 131)*. In Q fever endocarditis, fever has been recorded in 85% of patients, along with other systemic symptoms such as chills, headache, myalgias, and weight loss. Other complications resulting from Q fever endocarditis include congestive heart failure (76%), splenomegaly (42%), hepatomegaly (41%), clubbing (21%), and cutaneous signs, often the result of a leukocytoclastic vasculitis (22%).

23.7.2 Treatment of Q Fever

In the treatment of Q fever, the use of tetracyclines has been the preferred therapy. When initiated within the first 3 days of illness, treatment with tetracyclines shortens the duration of disease. Doxycycline has been the treatment of choice for acute Q fever—a dose of 100 mg of oral doxycycline twice daily for 15 to 21 days is the most frequently prescribed therapy.

Macrolide antibiotics, such as erythromycin, have also proved effective for the treatment of acute Q fever *(139)*.

Chronic Q fever endocarditis is much more difficult to treat effectively and often requires the use of multiple drugs. Two different treatments protocols have been evaluated: (i) doxycycline in combination with quinolones for at least 4 years; and (ii) doxycycline in combination with hydroxychloroquine for 1.5 to 3 years. The second regimen leads to fewer relapses but requires routine eye examinations to detect accumulation of chloroquine. Surgery to remove damaged valves may be required in some cases of *C. burnetii* endocarditis (http://cdc.gov/ncidod/dvrd/qfever).

23.7.2.1 Vaccines

An effective Q fever vaccine was developed in 1948. This preparation, consisting of formalin-killed and ether-extracted *C. burnetii* containing 10% yolk sac, was effective in protecting human volunteers from aerosol challenge *(140)*.

Vaccines prepared from phase I microorganisms, which were found to be 100 to 300 times more potent than phase II vaccines, form the basis for most current Q fever vaccines *(130)*. Purification methods have been improved over the years to better separate bacterial cells from egg proteins and lipids. However, use of the early phase I cellular vaccines has frequently been accompanied by adverse reactions, including induration of the vaccination site or the formation of sterile abscesses or granulomas. In addition, administration of a cellular vaccine to persons previously infected may result in severe and persistent local reactions *(130)*.

Attempts to maintain the vaccine's efficacy while decreasing the potential for adverse reactions led to testing cell extract as vaccines. Although such extracted cellular antigens were less reactive than were the intact microorganisms after injection, they were also less effective as vaccines *(130)*.

For the most part, attenuated Q fever vaccines are not used. However, a phase II attenuated strain, designated M-44, was developed in the former Soviet Union and has been used since 1960. This vaccine was shown to cause myocarditis, hepatitis, liver necrosis, granuloma formation, and splenitis in guinea pigs *(141)*.

In the late 1970s, a new CMR (chloroform-methanol residue) Q fever vaccine was developed. Initial testing has shown that the CMR vaccine did not cause adverse reactions in mice at doses several times larger than doses of phase I cellular vaccine. In addition, CMR vaccine did reduce the shedding of *C. burnetii* when used to vaccinate sheep *(130)*.

The most thoroughly tested Q fever vaccine in use today is Q-Vax. This is a formalin-killed, Henzerling strain, phase I cellular vaccine produced and licensed for use in Australia *(142)*. Q-Vax has been used successfully to prevent clinical Q fever in occupationally at-risk individuals. Thus, when a single subcutaneous dose (30 μg) of this vaccine was given to more than 2,000 abattoir workers screened for prior immunity, the protective efficacy was 100% *(142)*. The duration of protection was more than 5 years. The currently available Q fever vaccines would be of benefit to those occupationally at risk for Q fever, to those in areas endemic for Q fever, as well as to military and civilian populations who might be exposed as a result of a bioterrorist or biowarfare attack *(130)*.

23.8 Glanders

The causative agents of glanders and melioidosis are the nonfermenting Gram-negative bacilli *Burkholderia mallei* and *Burkholderia pseudomallei*, respectively.

Glanders is a disease of antiquity that has followed human civilization and has been described in writings by ancient Greek and Roman writers. The disease symptoms were recorded by Hippocrates around the year 425 BC, and the disease was given the name *melis* by Aristotle in approximately 350 BC.

Glanders (also known as equine, farcy, malleus, and droes) is naturally found in equines (horses, mules, and donkeys), and could occasionally be transmitted to humans *(143, 144)*. In addition, glanders can also be naturally contracted by goats, dogs, lions, and cats.

Through much of recorded history, glanders has been a world problem because horses and mules were vital means of transportation. The military also used them for moving supplies and troops and in battles among cavalries.

Only when horses were replaced by motorized transport in the early 20th century did the incidence of glanders decrease. Critical factors in reducing glanders in the Western world were the development of an effective skin test, a process of identification, and slaughter of infected animals. In the United States, glanders was eradicated in 1934 and has not been seen since 1945 *(143)*. Although developed nations have essentially been free of glanders, the disease is still commonly seen among domestic animals in Africa, Asia, the Middle East, and Central and South America (http://cdc.gov/ncidod/dbmd/diseaseinfo/glandrs_g.htm).

In World War I, *B. mallei* was used as biological weapon for the first time. The Central Powers infected Russian equines, causing disruption of supply lines and associated human deaths. Later, the Japanese used *B. mallei* against the Chinese, and there is anecdotal evidence that the former Soviet Union attempted to weaponize the pathogen and use it in Afghanistan (http://pathema.tigr.org/pathema/b_mallei.shtml).

The ease of transmission and severity of the disease have made *B. mallei* an obvious choice as an agent for biowarfare and bioterrorism, leading NIAID to categorize *B. mallei* as a category B Biological Disease *(145)*.

23.8.1 *Burkholderia mallei*

Burkholderia mallei (also previously known as *Pseudomonal mallei*, *Bacillus mallei*, *Pfeifferella mallei*, *Acinetobacter mallei*, *Loefferella mallei, Malleomyces mallei*, and *Acinetobacter mallei*) is a rod-shaped, nonmotile, obligate, Gram-negative bacillus that is an obligate animal pathogen, unlike the closely related *Burkholderia pseudomallei*, which can be found in tropical soil *(143)*. *Burkholderia mallei* was incorporated in its current genus in 1992 *(146)*. It produces an extracellular capsule that is an important virulence determinant and can survive drying for 2 to 3 weeks but is susceptible to heat (55°C for 10 minutes) and ultraviolet light.

In addition, *B. mallei* is susceptible to numerous disinfectants, including benzalkonium chloride, iodine, mercuric chloride in alcohol, potassium permanganate, 1% sodium hypochlorite, 70% ethanol, and 2% glutaraldehyde. It is less susceptible to phenolic disinfectants.

23.8.1.1 Genome Sequence of Burkholderia mallei

The complete genome sequence of *B. mallei* ATCC 23344, a highly pathogenic clinical isolate, has provided valuable insights into a number of putative virulence factors whose function was supported by comparative genome hybridization and expression profiling of the bacterium in hamster liver *in vivo (147)*. The genome consists of two chromosomes comprising approximately 5.7 million base pairs and an average G+C content of 68%. Its sequence is riddled with insertion sequences that have had a dramatic effect on its chromosomal structure.

The bioinformatics and laboratory analysis of the genome also provides further insight into the pathogenesis and biology of *B. mallei* and its relationship to the pathogenic *Burkholderia pseudomallei* and the nonpathogenic *B. thailandensis*.

The *B. mallei* genome was found to contain numerous insertion elements that have mediated extensive deletions and rearrangements of the genome relative to *B. pseudomallei*. The genome also contains a vast number of simple sequence repeats (more than 12,000). Furthermore, the observed variation in simple sequence repeats in key genes can provide a mechanism for generating antigenic variation, which may account for the mammalian host's inability to mount a durable adaptive response to a *B. mallei* infection *(147)*.

The location of the only virulence factors definitely shown to be essential for the pathogenicity of *B. mallei*, an extracellular capsule *(148)*, and a *Salmonella typhimurium*–like type III secretion system *(149)*, have been determined in the chromosome *(147)*.

In evolving from a metabolically versatile soil organism to a highly specialized obligate mammalian pathogen, structural flexibility appeared to be a major adaptive feature of the *B. mallei* genome.

23.8.1.2 Virulence of Burkholderia spp.

Better understanding of the *B. mallei* virulence determinants and pathogenesis will be critical for the development of

suitable vaccine candidates *(143)*. Some virulence factors of this pathogen have been characterized *(148, 149)*, and the recently completed genome of *B. mallei* ATCC 23344 (see Section 23.8.1.1) revealed putative gene products that were similar to virulence factors in other Gram-negative bacteria, including a type II secretion system, a type III secretion system, type IV pili, autotransporter proteins, iron acquisition proteins, fimbriae, quorum-sensing systems, and various transcriptional regulators *(150–152)*.

Capsule. Polysaccharide capsules represent highly hydrated polymers that mediate the interaction of bacteria with their immediate surroundings and often play integral roles in the interaction of pathogens with their hosts. A number of studies have provided clear evidence that *B. mallei* does form a capsule and that it is important for the pathogen's virulence *(143)*. Taken together, these studies have indicated that the *B. mallei* capsule may prevent phagocytosis early in the infection and may block the microbicidal action of the phagocytes after internalization. It is also possible that the capsule will confer resistance of the bacteria to lysosomal enzymes and will allow *B. mallei* to persist long enough to escape from the phagosome and/or phagolysosome, albeit by unknown mechanism. However, as of yet the chemical structure of the *B. mallei* capsule is unknown.

Subtractive hybridization has been used to identify genetic determinants present in *B. mallei (148)* and *B. pseudomallei (153)*. In both species, subtractive hybridization products have been mapped to a genetic locus encoding proteins involved in the biosynthesis, export, and translocation of a capsular polysaccharide *(148, 153)*. Furthermore, the *B. mallei* capsule gene cluster exhibited 99% nucleotide identity to a *B. pseudomallei* capsule gene cluster that encodes a homopolymeric surface polysaccharide *(148)*, and based on genetic and biochemical criteria, both *B. mallei* and *B. pseudomallei* gene clusters most closely resemble group 3 gene clusters because of their gene arrangement and because they lack the *kpsF* and *kpsU* homologues that are present in group 2 gene clusters *(148, 153)*. As shown in an immunogold electron micrograph study, *B. mallei* ATCC 23344 reacted with polyclonal capsular antibodies by forming a thick (approximately 200 nm) and evenly distributed surface layer around the bacteria.

Antigen 8. In several studies, an extracellular capsule-like substance called antigen 8 (Ag8) has been identified on the surface of both *B. mallei* and *B. pseudomallei* and is thought to be a pathogenicity factor because of its antiphagocytic and immunosuppressive properties *(154,154a,155)*. Ag8 is a glycoprotein composed of 10% protein and 90% carbohydrate and has a molecular mass of approximately 88 kDa *(154)*. In *B. mallei* cultures, Ag8 production was not detected until the second half of the exponential growth phase, and production was maximal during the stationary phase *(154)*. Because the carbohydrate moiety of Ag8, a homogeneous polymer of 6-d-*D*-mannoheptose *(155)*, was found to be identical in structure to the capsular polysaccharide of *B. pseudomallei* and *B. mallei*, further studies will be necessary to determine whether Ag8 and the capsule (see the preceding paragraph) are the same molecule or are distinct molecular moieties *(143)*.

Lipopolysaccharide (143). Similar to *Burkholderia pseudomallei (156)*, *B. mallei* strains deficient in LPS *O*-antigen are sensitive to killing by 30% normal human serum (NHS) and are less virulent than the wild-type strains *(157)*. Previous studies have revealed that *B. mallei* LPS *O*-antigens cross-react with polyclonal antibodies raised against *B. pseudomallei* LPS *O*-antigens *(48, 157, 158)*, and that the LPS *O*-antigen gene clusters of these species are 99% identical at the nucleotide level *(156, 157)*. In fact, the *B. mallei* LPS *O*-antigen was found to be similar to the previously described *B. pseudomallei* LPS *O*-antigen, a heteropolymer of repeating *D*-glucose and *L*-talose *(157, 159, 160)*. However, changes are apparent in the *O*-acetylation pattern of the *B. mallei* *L*-talose residue compared with the pattern in *B. pseudomallei*. Similar to the *B. pseudomallei* LPS *O*-antigen, the *B. mallei* LPS *O*-antigen contained an *O*-acetyl or *O*-methyl substitution at the 2′-position of the talose residue. On the other hand, the *B. mallei* LPS *O*-antigen is devoid of an *O*-acetyl group at the 4′-position of the talose residue *(157)*. Thus, the structure of *B. mallei* LPS *O*-antigen is best described as "3-β-*D*-glucopyranose-(1,3)-6-deoxy-α-*L*-talopyranose-(1-" in which the talose residue contains 2-*O*-methyl or 2-*O*-acetyl substituents *(157)*.

Pathoadaptive Mutations (143). Comparative genomic analysis of closely related bacteria has demonstrated that gene loss and gene inactivation are common themes in host-adapted pathogens *(161)*. These mutations, called pathoadaptive mutations, will improve fitness/virulence by modifying traits that interfere with survival in host tissues *(161)*. *B. mallei* is a host-adapted parasite of equines whereas *B. pseudomallei* is an opportunistic parasite of numerous hosts. The genomic sequences of *B. mallei* ATCC 23344 *(147)* and *B. pseudomallei (162)* have been completed and can be directly compared (see also Section 23.8.1.1). Although the genes conserved between these species were approximately 99% identical, *B. pseudomallei* contained approximately 1 megabase (Mb) of DNA that was not present in *B. mallei*. It is plausible that *B. pseudomallei* may have acquired this DNA through lateral transfer after the divergence of these two species from a common progenitor *(163)*. Alternatively, this DNA was present in the common progenitor and was subsequently deleted in *B. mallei (164)*. In addition, *B. mallei* has numerous insertion sequences, and several of these are present within genes (gene inactivation). Gene loss and gene inactivation probably played important roles in the evolution of *B. mallei* by eliminating factors that were not required (or

were inhibitory) for a successful host-pathogen interaction. Thus, it appears that pathoadaptive mutations have played an important role in the evolutionary adaptation of *B. mallei* to a parasitic mode of existence *(143)*.

Exopolysaccharide. A survey of the phenotypic traits that are present in *B. mallei* and *B. pseudomallei* (but absent in the nonpathogenic *B. thailandensis*) may allow for the identification of new virulence determinants *(143)*. One virulence factor that fits these criteria is a capsule-like exopolysaccharide *(165)*. This exopolysaccharide is a linear tetrasaccharide repeating unit consisting of three galactose residues, one bearing a 2-linked *O*-acetyl group and a 3-deoxy-*D*-manno-2-octulosonic acid residue *(166)*. However, the genes encoding the exopolysaccharide have not been identified in *B. mallei* and *B. thailandensis*, and its role in the pathogenesis is currently unknown.

23.8.2 Clinical Manifestations of Glanders in Humans

Humans can become infected with *B. mallei* after contact with sick animals or infectious materials. Transmission is typically through small wounds and abrasions in the skin. Ingestion or inhalation with invasion through the mucous membranes (nasal, oral, and conjunctival), eyes, and by inhalation into the lungs is also possible. Cases are usually seen in people who handle laboratory samples *(167)* or who have frequent close contact with equines.

Case fatality rate is 95% for untreated septicemic infections and more than 50% with traditional antibiotic treatment, although susceptibility data suggest that newer antibiotics should be effective.

In natural infections, the incubation period is 1 to 14 days. Infections from aerosolized forms in biological weapons are expected to have an incubation period of 10 to 14 days.

In humans, the clinical symptoms of glanders depend on the route of infection with the organism. The types of infection include localized, pus-forming cutaneous infections, pulmonary infections, bloodstream (septicemic) infections, and chronic suppurative infections of the skin. *Combinations of syndromes can occur*. Generalized symptoms of glanders include fever, muscle pain, chest pain, muscle tightness, and headache. Additional symptoms have included excessive tearing of the eyes, light sensitivity, and diarrhea (http://cdc.gov/ncidod/dbmd/diseaseinfo/glandrs_g.htm).

Localized Infections. If there is a cut or scratch in the skin, a localized infection with ulceration will develop within 1 to 5 days at the site where the bacteria entered the body. Swollen lymph nodes may also be present. Infections (ulcers) involving the mucous membranes in the eyes, nose, and respiratory tract will cause increased mucus production and blood-tinged discharge from the affected sites. Mucosal or skin infections can disseminate; symptoms of disseminated infections include a popular or pustular rash, abscesses in the internal organs (particularly the liver and spleen), and pulmonary lesions. Disseminated infections are associated with septic shock and high mortality.

Pulmonary Infections. In pulmonary infections, pneumonia, pulmonary abscesses, and pleural effusion can also occur. Chest x-rays will show localized infection in the lobes of the lungs. Symptoms include cough, fever, dyspnea, and mucopurulent discharge. Skin abscesses sometimes develop after several months.

Bloodstream (Septicemic) Infections. Glanders bloodstream infections are usually fatal within 7 to 10 days. In the septicemic form, fever, chills, myalgia, and pleuritic pain develop acutely. Other symptoms may include generalized erythroderma, jaundice, photophobia, lacrimation, diarrhea, and granulomatous or necrotizing lesions. Tachycardia, cervical adenopathy, and mild hepatomegaly or splenomegaly may also be observed.

In the septicemic form, blood cultures of *B. mallei* may be negative just before death.

Chronic Infections. The chronic form of glanders involves multiple abscesses within the muscles of the arms and legs or in the spleen or liver.

23.8.3 Treatment of Glanders

Because *B. mallei* is considered a potential biological weapon and its intentional release has already been documented *(143, 168)*, there is a clear need for effective treatment strategies of human glanders as well as postexposure prophylaxis.

Because *B. mallei* is variably susceptible to antibiotics *(169, 171)*, long-term treatment or multiple drugs may be necessary. However, treatment may be ineffective, especially in septicemia, because of the pathogen's *intrinsic resistance* to a wide range of antimicrobial agents including β-lactam antibiotics, aminoglycosides, and macrolides *(170, 171)*.

The antibiotic susceptibility pattern profile of *B. mallei* has shown *in vitro* susceptibility to ceftazidime, imipenem, meropenem, and doxycycline. Although initial treatment with imipenem or meropenem, or with ceftazidime for 2 to 3 weeks has been recommended, there is little experience in treating glanders in humans. In severe cases, a combination therapy with doxycycline or co-trimoxazole may be considered *(172, 173)*. In mild cases, initial oral therapy with an antimicrobial agent (doxycycline, co-amoxiclav, fluoroquinolones, TMP-SMZ) may be sufficient *(174)*.

Dose Regimens (www.emea.eu.int/pdfs/human/bioterror/10.glandersmelioidosis.pdf)

(i) *Imipenem*. In adults: 50 mg/kg daily, up to 1.0 g, 4 times daily. In children over 3 years of age: 15 mg/kg, 4 times daily (up to maximum of 2.0 g daily); over 40 kg, use adult dose. In children between 3 months and 3 years: 15 to 25 mg/kg, 4 times daily. In pregnancy, same regimens as in nonpregnant adults should be considered; it is recommended when possible to cease breastfeeding *(174)*.
(ii) *Meropenem*. In adults: 500 to 1,000 mg, intravenously, 3 times daily. In children over 3 months: 10 to 20 mg/kg, 3 times daily; adult dose for 50 kg and over. In pregnancy, same regimens as in nonpregnant adults should be considered; it is recommended when possible to cease breastfeeding *(174)*.
(iii) *Ceftazidime*. In adults: 2.0 g, intravenously, 3 times daily. In children over 2 months: 100 mg/kg daily in 3 divided doses (maximum dose is 6.0 g). In children younger than 2 months: 60 mg/kg daily in 2 divided doses. In pregnancy, same regimens as in nonpregnant adults should be considered; it is recommended when possible to cease breastfeeding *(174)*.
(iv) *Doxycycline*. In adults: 100 mg, intravenously, twice daily. In children over age 8 and over 45 kg weight: use the adult dose. In children over 8 years and less than 45 kg weight: 2.2 mg/kg, intravenously, twice daily. In children younger than 8: 2.2 mg/kg, intravenously, twice daily. Maximum dose 200 mg per day. In pregnancy, same regimens as in nonpregnant adults should be considered; it is recommended when possible to cease breastfeeding *(174)*.
(v) *TMP-SMX (trimethoprim-sulfamethoxazole)*. In adults: TMP: 6 to 8 mg/kg daily and SMX: 40 mg/kg daily, intravenously, in 1 or 2 divided doses, followed by TMP: 6 to 8 mg/kg daily and SMX: 40 mg/kg daily orally in 1 or 2 divided doses. Maximum total dose is 1,440 mg, twice daily. Consideration could be given to reducing the dose after 2 weeks *(174)*.
In children younger than 8: TMP: 6 to 8 mg/kg daily and SMX: 30 to 40 mg/kg daily, intravenously in 2 divided doses, followed by TMP: 6 to 8 mg/kg daily and SMX: 30 to 40 mg/kg daily, orally in 1 or 2 divided doses. Consideration could be given to reducing the dose after 2 weeks. In pregnancy, same regimens as in nonpregnant adults should be considered; it is recommended when possible to cease breastfeeding *(174)*.

23.8.3.1 Vaccines

Currently, there is no evidence for immunity against glanders by virtue of previous infection or vaccination *(143, 175)*. Infections in horses that seemed to recover symptomatically from glanders would recrudesce when the animals were challenged with *B. mallei*. Numerous attempts to vaccinate horses and laboratory animals against glanders were unsuccessful during the period 1895–1928 *(143)*. Even though vaccination resulted in some resistance to infection, the animals still contracted glanders.

23.9 Melioidosis

Melioidosis, also called *Whitmore's disease*, is an infection caused by *Burkholderia pseudomallei*, a Gram-negative bacterium closely related to *B. mallei*, the etiologic agent of glanders (see Section 23.8). Melioidosis is clinically and pathologically similar to glanders, but the ecology and the epidemiology of melioidosis are different from those of glanders. Melioidosis is predominately a disease of tropical climates, and the bacterium that causes melioidosis is found in contaminated water and soil. It is spread to humans and animals through direct contact with the contaminated source. Glanders is contracted by humans from infected domestic animals or by inhalation *(176)* (http://www.cdc.gov/ncidod/dbmd/diseaseinfo/melioidosis_g.htm).

B. pseudomallei is an organism that has been considered as a potential agent for biological warfare and bioterrorism. Melioidosis is endemic to Southeast Asia, with the highest concentration of cases in Vietnam, Cambodia, Laos, Thailand, Malaysia, Myanmar (Burma), and northern Australia. In addition, it is also seen in the South Pacific, Africa, India, and the Middle East. The bacterium is so prevalent that it has been isolated from troops of all nationalities that have served in areas of endemic disease. A few isolated cases of melioidosis have occurred in the Western Hemisphere, in Mexico, Panama, Ecuador, Haiti, Peru, Guyana, and in the states of Hawaii and Georgia. In the United States, confirmed cases range from none to five each year and occur among travelers and immigrants. Since the infamous "Affaire du Jardin des Plantes," in which a panda donated by Mao Ze Dong to French President Pompidou was the index case in an epidemic of melioidosis that decimated the large animals of the Paris zoological gardens *(176, 177)*, *B. pseudomallei* has also emerged as a major veterinary pathogen. Animals susceptible to melioidosis include sheep, goats, horses, swine, cattle, dogs, and cats. Transmission occurs by direct contact with contaminated soil and surface waters. In Southeast Asia, the pathogen has been repeatedly isolated from agricultural fields, with infection occurring primarily during the rainy season. Humans and animals are believed to acquire the infection by inhaling dust, ingesting contaminated water, and having contact with contaminated soil, especially through skin abrasions, and for military troops by contamination of war wounds. Person-to-person transmission can also occur. Two cases of sexual transmission have been reported—the transmission in both cases was preceded by a clinical history of chronic prostatitis

in the source patient (http://www.cdc.gov/ncidod/dbmd/diseaseinfo/melioidosis_g.htm).

In endemic areas, melioidosis is a disease of the rainy season and affects mainly people with underlying predisposition to infection, such as those with diabetes, renal disease, cirrhosis, thalassemia, or alcoholism, as well as those who are immunosuppressed *(176)*. However, melioidosis seems not to be associated with HIV infection. In addition, melioidosis has been recognized as a possible cause of chronic infection in patients with cystic fibrosis *(178, 179)*.

23.9.1 Burkholderia pseudomallei

Burkholderia pseudomallei, the etiologic agent of melioidosis, is a motile, aerobic, nonspore-forming, Gram-negative bacterium. It is a soil saprophyte and can be recovered readily from water and wet soils in rice paddies in endemic areas *(176)*.

The genome of *B. pseudomallei* was recently sequenced and found to be relatively large, 7.24 megabase (Mb) pairs *(161)*. It is unequally divided between two chromosomes (4.07 and 3.17 Mb) with a G+C content of 68%, and a significant functional partitioning between them. The larger chromosome encodes many of the core functions associated with central metabolism and cell growth, whereas the smaller chromosome carries more accessory functions associated with adaptation and survival in different niches. Genomic comparisons with closely (see Section 23.8.1.1) and more distantly related bacteria revealed a greater level of gene order conservation and a greater number of orthologous genes on the large chromosome, suggesting that the two replicons have distinct evolutionary origins *(161)*.

One striking feature of the *B. pseudomallei* genome was the presence of 16 genomic islands that together made up 6.1% of the genome. Further analysis revealed that these islands were variably present in a collection of invasive and soil isolates but entirely absent from the clonally related organism *B. mallei*; it has been hypothesized that variable horizontal gene acquisition by *B. pseudomallei* is an important feature of recent genetic evolution and that this has resulted in a genetically diverse pathogenic species *(161)*.

23.9.2 Pathogenesis and Virulence Determinants of Burkholderia pseudomallei

B. pseudomallei, like many soil bacteria, is difficult to kill. It can survive in triple-distilled water for years *(143)*. It is resistant to complement, lysosomal defensins, and cationic peptides, and it produces proteases, lipase, lecithinase, catalase, peroxidase, superoxide dismutase, hemolysins, a cytotoxic exolipid, and at least one siderophore *(180–183)*. Furthermore, the pathogen can survive inside several eukaryotic cell lines and is seen within phagocytic cells in pathologic specimens *(183–185)*. After internalization, the pathogen escapes from the endocytic vacuoles into the infected cell cytoplasm and then forms membrane protrusions by inducing actin polymerization at one pole. The actin protrusions from the infected cell membrane mediate spread of the organism from cell to cell *(186)*. *B. pseudomallei* contains several type III secretion systems that play an important role both in its spread and in intracellular survival *(185, 186)*.

The role of the exotoxins in the pathogenesis of melioidosis is still unresolved *(176)*. The high mortality of melioidosis is related to an increased propensity to develop high bacteremias (> 1 CFU/mL), but the relation between bacterial counts in blood and mortality is similar to that of other Gram-negative bacteria *(187)*. This finding suggests that exotoxins do not contribute directly to the outcome.

The cell wall LPS, which is the immunodominant antigen, is highly conserved. In addition, *B. pseudomallei* produces a highly hydrated glycocalyx polysaccharide capsule *(188)*, an important virulence determinant *(189)* that helps to form slime. This capsule facilitates the formation of microcolonies in which the organism is both protected from antibiotic penetration and phenotypically altered, resulting in reduced susceptibility to antibiotics (small colony variants) *(190)*. Passive immunization with antibody to this exopolysaccharide reduced the lethality in mice *(191)*. To date, the organisms that cause invasive disease are indistinguishable from those found in the environment *(176)*.

The position-308 promoter region of the TNF-α gene has been associated with polymorphism (TNF2 allele) that is related to acquisition of melioidosis and disease severity *(192)*. Furthermore, melioidosis has been positively associated with HLA class II DRB1*1602 in Thailand *(193, 193a)*. This allele was associated significantly with septicemic melioidosis, whereas DQA1*03 was negatively associated; this association was independent of confounders such as diabetes mellitus *(193)*.

23.9.3 Clinical Manifestations of Melioidosis

Generally, melioidosis presents as a febrile illness ranging from an acute fulminant septicemia to a chronic debilitating localized infection. The majority of patients are septicemic *(176)* (http://www.cdc.gov/ncidod/dgmd/diseaseinfo/melioidosis_g.htm). The disease can be categorized as acute or localized infection, acute pulmonary infection, acute bloodstream (septicemic) infection, and chronic suppurative infection. Asymptomatic infection is also possible.

Melioidosis can spread from person to person by contact with the blood and body fluids of an infected person. Two documented cases of male-to-female sexual transmission have involved males with chronic prostatic infection due to melioidosis. The incubation period of melioidosis is not clearly defined but may range between 2 days and many years (http://www.cdc.gov/ncidod/dgmd/diseaseinfo/melioidosis_g.htm).

Acute Localized Infection. This form is generally localized as a nodule and results from inoculation through a break in the skin. Symptoms include fever, general muscle pain, and may progress rapidly to infection of the bloodstream.

Pulmonary Infection. The lung is the most commonly affected organ, either presenting with cough and fever resulting from a primary lung abscess or pneumonia, or secondary to septicemic spread (blood-borne pneumonia). The clinical picture of the disease may be characterized from mild to severe pneumonia. The onset is typically accompanied by high fever, headache, anorexia, and general muscle soreness. Chest pain is common, but a nonproductive or productive cough with normal sputum is the hallmark of pulmonary melioidosis. However, sputum may also be purulent but rarely blood-stained. Large or peripheral lung abscesses may rupture into the pleural space to cause empyema *(176)*.

Acute Bloodstream Infection. Patients with underlying disease such as diabetes, HIV infection, and renal failure are affected by this form of melioidosis, which usually will result in septic shock. The symptoms of the bloodstream infection vary depending on the site of the original infection, but in general, they will include respiratory distress, severe headache, fever, diarrhea, development of pus-filled lesions on the skin, muscle tenderness, and disorientation. This is typically an infection of short duration, and abscesses will be found throughout the body.

Chronic Suppurative Infection. Chronic melioidosis is an infection that involves the organs of the body. Seeding and abscess formation may arise in any organ, although the joints, viscera, lymph nodes, skin, brain, liver, spleen, skeletal muscle, and the prostate are common sites. Renal abscesses are often associated with calculi and urinary infection. Corneal ulcers secondary to trauma and then exposure to contaminated water can be rapidly destructive *(193a)*.

23.9.4 Treatment of Melioidosis

Melioidosis is difficult to treat, and the initial intensive care management of severe illness includes resuscitation of patients with adequate intravenous fluids, because hypovolemia is common in the acute phase, as well as administration of high doses of parenteral antibiotics *(176)*. The antibiotic of choice is ceftazidime *(194, 195)*. Other third-generation cephalosporins were less effective. Carbapenems kill *B. pseudomallei* more rapidly than do cephalosporins *(196)*, and in a large randomized trial imipenem proved equivalent to ceftazidime *(197)*.

Antibiotic combinations have also been used as empirical treatment of melioidosis including parenteral amoxicillin-clavulanate and cefoperazone-sulbactam; however, such treatment should be changed to ceftazidime or a carbapenem once the diagnosis of melioidosis has been confirmed *(176)*.

The risk of relapse has been related to the adherence to treatment and the initial extent of the disease, but not to the underlying condition *(176)*. The prognosis of melioidosis has been much better in children than in adults, and relapse was rarely observed. Adult patients require follow-up observation throughout their lives *(176)*.

Parenteral Treatment. Because the therapeutic response is very slow, parenteral treatment should be given for at least 10 days and should continue until improvement is noted and the patient can take oral drugs. Dose regimens often need to be adjusted for renal failure *(176)*.

Ceftazidime is usually given intravenously at 40 mg/kg every 8 h for a total of 120 mg/kg daily, or at 19 mg/kg intravenously immediately followed by a continuous infusion of 3 to 5 mg kg^{-1} h^{-1}. Other third-generation cephalosporins (e.g., cefotaxime, ceftriaxone) should not be used, because these antibiotics have been associated with increased mortality despite evidence of acceptable *in vitro* susceptibility *(176)*.

Imipenem is administered intravenously at 20 mg/kg every 8 hours for a total of 60 mg/kg daily *(176)*.

Intravenous amoxicillin-clavulanate is effective as empirical treatment of suspected septicemia at doses of 27 mg/kg given every 4 h (*not every* 8 h) for a total of 162 mg/kg daily *(176)*.

Oral Treatment. Oral treatment should be administered to complete a full 20 weeks of treatment. In adults, the oral treatment of choice is a four-drug combination of chloramphenicol (40 mg/kg daily in 4 divided doses), doxycycline (4 mg/kg daily in 2 divided doses), and trimethoprim-sulfamethoxazole (10 mg and 50 mg/kg daily, respectively, in 2 divided doses). *Note: chloramphenicol should be given only for the first 8 weeks*.

In children (8 years of age) and pregnant women, the recommended drug regimen comprises amoxicillin-clavulanate (amoxicillin 30 mg/kg daily; clavulanic acid 15 mg/kg daily) plus amoxicillin (30 mg/kg daily) *(176)*.

23.9.5 Melioidosis Acute Suppurative Parotitis

Melioidosis acute suppurative parotitis is a unique syndrome that occurs mainly in children in Southeast Asia without any other evidence of an underlying predisposing condition

(198). The syndrome is unusual in Australia. Patients present with fever, pain, and swelling over the parotid gland. In about 10% of cases, parotitis is bilateral. In advanced cases, rupture can arise, either to the skin or through the external ear *(198)*.

This condition has been managed with antibiotics (initially ceftazidime, followed by oral amoxicillin-clavulanate) and with incision and drainage, with great care applied not to damage the facial nerve. Delay in drainage can result in permanent Bell's palsy. The syndrome is usually managed with 8 weeks of treatment *(176)*.

23.9.6 Brain-Stem Encephalitis

In about 4% of cases from Australia and rarely elsewhere, melioidosis presents as brain-stem encephalitis with peripheral motor weakness of flaccid paraparesis *(199–201)*. Prominent features of this neurologic syndrome include unilateral limb weakness, cerebellar signs, and cranial nerve palsies. The pathogenesis of this condition has been uncertain, but new evidence has suggested that multiple focal microabscesses in the brain stem and spinal cord have been the cause. Antibiotic treatment has been similar to that given for other forms of melioidosis *(176)*.

23.10 Viral Hemorrhagic Fever: Filoviruses

The filoviruses are enveloped particles classified in the family Filoviridae, and together with two other families (Paramyxoviridae and Rhabdoviridae) they belong in the order Mononegavirales. Within the family Filoviridae, there is a single genus, Filovirus, that is separated into two sero-/genotypes, Marburg (MARV) and Ebola (EBOV). Ebola is further subdivided into four subtypes: Zaire, Sudan, Reston, and Ivory Coast (http://www.cdc.ncidod/dvrd/spb/mnpages/dispages/filoviruses.htm).

Structurally, Filovirus virions (complete viral particles) may appear in several shapes, a biological feature known as pleomorphism. These shapes include long, sometimes branched filaments, as well as shorter filaments shaped like a "6," or "U," or a circle. Viral filaments may measure up to 14,000 nm in length, have a uniform diameter of 80 nm, and are enveloped in a lipid membrane. Each virion contains one molecule of single-stranded, negative-sense RNA, approximately 19 kb long. New viral particles are created by budding from the surface of their host's cells. However, the Filovirus replication pathways are not well understood.

The first Filovirus was recognized in 1967 when a number of laboratory workers in Germany and the former Republic of Yugoslavia, who were handling tissues of green monkeys (*Cercopithecus aethiops*), developed hemorrhagic fever. A total of 31 cases and seven deaths were associated with these outbreaks. The virus was named for the city of Marburg, Germany, the site of one of the outbreaks. After the initial outbreaks, the virus disappeared. It did not re-emerge until 1975, when a traveler, most likely exposed in Zimbabwe, became ill. A few sporadic cases of Marburg hemorrhagic fever have been identified since that time.

The Ebola virus was first identified in 1976 when two outbreaks of Ebola hemorrhagic fever (Ebola HF) occurred in northern Zaire (Democratic Republic of Congo) and southern Sudan *(202–207)*. These two outbreaks eventually involved two different species of Ebola virus; both were named after the nations in which they were first discovered. Both the Zaire and Sudan subtypes proved to be highly lethal, as 90% of the Zairian cases and 50% of the Sudanese cases resulted in death. Since 1976, the Ebola virus has sporadically appeared in Africa, with small to midsize outbreaks confirmed between 1976 and 1979. Large epidemics of Ebola HF occurred in Kikwit, Zaire, in 1995 and in Gulu, Uganda, in 2000. Smaller outbreaks were identified in Gabon between 1994 and 1996 (http://www.cdc.ncidod/dvrd/spb/mnpages/dispages/filoviruses.htm). The Ebola-Reston subtype was isolated from cynomolgus monkeys (*Macaca fascicularis*) imported from the Philippines into the United States in 1989 and into Italy in 1992. The Ebola–Ivory Coast subtype emerged on the Ivory Coast in 1994 *(208, 209)*.

Epidemiology. It appears that the filoviruses are zoonotic because they are transmitted to humans from ongoing life cycles in animals other than humans. The Ebola-Reston subtype is the only known Filovirus that does not cause severe disease in humans; however, it can be fatal to monkeys *(210, 211)*. In spite of numerous attempts to locate the natural reservoir(s) of Ebola and Marburg viruses, their origins remain undetermined. However, because the virus can be replicated in some species of bats, some bat species native to the areas where the viruses are found may prove to be their carriers (http://www.cdc.ncidod/dvrd/spb/mnpages/dispages/filoviruses.htm).

Just how the virus is transmitted to humans is not known. However, once a human is infected, person-to-person transmission is the means by which further infections occur. Usually, transmission involves close contact between an infected individual or his or her body fluids and another person. During recorded outbreaks of hemorrhagic fever caused by Filovirus infection, persons who cared for or worked very closely with infected patients were especially at risk of becoming infected themselves *(212)*. Nosocomial transmission through contact with infected body fluids (reuse of unsterilized syringes, needles, or other contaminated material) has also been an important factor in spreading the disease. Although the viruses display some

capability of infection through small-particle aerosols, airborne spread among humans has not been clearly demonstrated (http://www.cdc.ncidod/dvrd/spb/mnpages/dispages/filoviruses.htm).

In conjunction with the WHO, the CDC has developed practical, hospital-based guidelines, titled *Infection Control for Viral Haemorrhagic Fevers in the African Health Care Setting* (available at the CDC Web site under Ebola Hemorrhagic Fever) to help health care facilities recognize cases and prevent further hospital-based transmission of disease, using locally available materials and few financial resources.

23.10.1 Genome Structure of Filoviruses

The genomes of Ebola and Marburg consist of a single, negative-stranded, RNA linear molecule and are approximately 19 kb long (Marburg, 19.1 kb; Ebola, 18.9 kb) *(213)* (see also http://www.gsbs.utmb.edu/microbook/ch072.htm). The RNA is noninfectious, not polyadenylated, and complementary to polyadenylated viral subgenomic RNA species. The genes have been defined by highly conserved transcriptional start and termination signals at their 3′ and 5′ ends. Some genes overlap, but the positions and numbers of overlaps differ between the two types of viruses. The length of the overlaps is limited to five highly conserved nucleotides within the transcriptional signals. Most genes tended to possess long noncoding sequences at their 3′ and/or 5′ ends, which have contributed to the increased length of the genome. The genomes were complementary at the very extreme ends.

The filoviruses' nucleoprotein (NP) is the major structural phosphoprotein, and only the phosphorylated form is incorporated into the virions, as demonstrated for Marburg. The NP is the major component of the ribonucleoprotein complex (RNP). The RNP consists of the nonsegmented negative-stranded RNA genome and four of the structural proteins: nucleoprotein (NP); virion structural protein (VP) 30; VP35; and L (large or polymerase) protein. Two other virion proteins, VP24 and VP40, are membrane-associated, and the spikes are formed by the glycoprotein (GP).

23.10.2 Pathogenesis of Filoviruses

The fatal outcome in filoviral infection is associated with an early reduction in the number of circulating T cells, the host's failure to develop specific humoral immunity, and the release of proinflammatory cytokines *(214–217)*.

The lack of evidence for massive direct vascular involvement in infected hosts supports the role of active mediator molecules in the pathogenesis of the disease. Although the source of these mediators during Filovirus infections is still unknown, candidate cells exist. Besides the endothelium, the common denominator remains the macrophage, which is known as a pivotal source of a different protease, H_2O_2, and mediators such as tumor necrosis factor (TNF)-α. The latter finding supports the concept of a mediator-induced vascular instability, thus suggesting that increased permeability may be a key mechanism for the development of the shock syndrome in severe and fatal cases. The tendency toward bleeding could be the result of endothelial damage caused directly by virus replication, as well as indirectly by cytokine-mediated processes. Furthermore, the combination of viral replication in endothelial cells and the virus-induced cytokine release from macrophages may also promote a distinct proinflammatory endothelial phenotype that can trigger the coagulation cascade (http://www.gsbs.utmb.edu/microbook/ch072.htm).

Clinical and biochemical findings have supported the anatomic observations of extensive liver involvement, renal damage, changes in vascular permeability, and activation of the clotting cascade (http://www.gsbs.utmb.edu/microbook/ch072.htm). Necrosis of visceral organs is the consequence of virus replication in parenchymal cells. However, no organ is sufficiently damaged to cause death. Fluid distribution problems and platelet abnormalities indicate dysfunction of endothelial cells and platelets. The shock syndrome in severe and fatal cases seems to be mediated by virus-induced release of humoral factors such as cytokines. The Filovirus glucoprotein is believed to carry an immunosuppressive domain, and immunosuppression has been observed in monkeys (http://www.gsbs.utmb.edu/microbook/ch072.htm).

Recent experimental data have shown that some of the antibodies produced during Ebola virus infection have enhanced its infectivity, and this enhancement was mediated by antibodies to the viral glycoprotein and by complement component C1q. This finding suggested a novel mechanism of antibody-dependent enhancement (ADE) of virus infection. Cross-links between virus and cell, via antibodies, C1q, and C1q ligands at the cell surface may promote either binding of the virus to Ebola virus–specific receptors or endocytosis of the target cells, suggesting that ADE of infectivity may account for the extreme virulence of Ebola virus. Furthermore, the human macrophage galactose- and *N*-acetyl galactosamine-specific C-type lectins enhanced the infectivity of the filoviruses. Interestingly, these C-type lectins are present on cells known to be major Filovirus targets (e.g., macrophage hepatocytes and dendritic cells), suggesting a role for these C-type lectins in viral tissue tropism *in vivo*. The overall results led to a hypothesis suggesting that the filoviruses may use antibodies, complement components,

and C-type lectins to gain cellular entry, depending on the cell type, and promote efficient viral replication (http://www.czc.hokudai.ac.jp/epidemiol/research_e.html).

23.10.2.1 Filoviral Glycoprotein

The membrane-anchored Filovirus glycoprotein (GP) is attached on the surface of virions and infected cells. It mediates receptor binding and fusion. The Filovirus glycoproteins are considered to be major determinants of viral pathogenicity and contribute to both immunosuppression and vascular dysregulation *(214, 218–221)*.

The transmembrane glycoproteins of many animal and human retroviruses share structural features, including a conserved region that has strong immunosuppressive properties *(222, 223)*. As a result, CKS17, a synthetic peptide corresponding with the conserved region in oncogenic retroviruses, has been used to study the pathophysiology of immunosuppression *(224, 225)*. In particular, CKS17 causes an imbalance of human type-1 and type-2 cytokine production, suppresses the cell-mediated immunity *(226)*, and blocks the activity of protein kinase C, a cellular messenger involved in T-cell activation *(227, 228)*. Furthermore, it has been determined that there is a region of strong secondary structure conservation between the *C*-terminal domain of the envelope glycoprotein of the filoviruses and CKS17 *(214)*. Thus, a 17-amino-acid domain in the filoviral glycoproteins was shown to resemble an immunosuppressive motif in the retroviral envelope proteins; that is, dysregulating the Th-1 and Th2 responses and depleting $CD4^+$ and $CD8^+$ T-cells through apoptosis. Moreover, an alignment of the filoviral glycoprotein and the retroviral immunosuppressive domains illustrated primary sequence similarity between a wide range of retroviruses and filoviruses. Three cysteine residues implicated in disulfide bonding were also conserved, reinforcing similarities in secondary structure. Functional analysis of the putative immunosuppressive domains in various species of Ebola and Marburg has demonstrated that the immunosuppressive effect of different species of the GP peptides was consistent with the pathogenicity observed in different animal hosts *(214)*. These and other findings have also been consistent with a previous observation that the Ebola-Reston subtype was not pathogenic in humans *(221)* and may enable the development of specific strategies to reduce the extreme morbidity and mortality associated with hemorrhagic fever due to the Ebola and Marburg filoviruses *(214)*.

23.10.2.2 Filovirus Replication

It is thought that the cell entry of the filoviruses is mediated by the GP as the only surface protein of virion particles. Studies on Marburg (strain Musoke) infection of hepatocytes have identified the asialoglycoprotein receptor as a receptor candidate *(229)*. It is not known if the next step in the virus entry involves a fusion process at the plasma membrane or fusion after endocytosis of virus particles. Neither has the uncoating mechanism been studied. The Filovirus transcription and replication take place in the cytoplasm of the infected cells. The role of gene overlaps in regulating transcription is not known, but transcription may be reinitiated by repositioning of the polymerase at the downstream start site (back-up mechanism). Alternatively, the polymerase may occasionally terminate transcription at the overlap and initiate transcription of the downstream gene without polyadenylation of the upstream gene. The switch mechanism between transcription and replication has not been studied. Virions usually bud at the plasma membrane. Mature particles usually exit preferentially in a vertical mode, but budding via the longitudinal axis has also been observed *(229)*.

With the Ebola GP gene, the transcription occurs from two open reading frames *(229)*. The primary gene product is a small nonstructural glycoprotein that is secreted from the infected cells. To express the full-length GP, two independent mechanisms are possible: transcriptional editing of a single nucleotide at a run of uridine residues; or translational frame shifting (-1) at or just past the editing site of unedited transcripts. The Marburg GP, however, is expressed in a single frame, and the gene does not contain sequences favoring mechanisms such as editing or frame shifting *(229)*.

23.10.3 Hemorrhagic Fever: Clinical Manifestations

The filoviruses cause a severe hemorrhagic fever in humans and non-human primates *(229)*. The onset of disease is very sudden, with fever, chills, headache, myalgia, and anorexia. These symptoms may be followed by abdominal pain, sore throat, nausea, vomiting, cough, arthralgia, diarrhea, and pharyngeal and conjunctival vasodilation. Patients are dehydrated, apathetic, and disoriented. Further symptoms include the development of characteristic, nonpruritic, maculopapular centripetal rash associated with varying degree of erythema, which desquamates by day 5 or 7 of the illness *(229)*.

Hemorrhagic manifestations will develop at the peak of the illness and are of prognostic value. Bleeding into the gastrointestinal tract is the most prominent, in addition to petechia and hemorrhages from puncture wounds and mucous membranes. The laboratory parameters, although less characteristic, include leukopenia (as low as $1,000/\mu L$); left shift with atypical lymphocytes; thrombocytopenia (50,000 to $100,000/\mu L$); markedly elevated

serum transaminase levels (typically aspartate aminotransferase exceeding alanine aminotransferase); hyperproteinemia; and proteinuria.

There is a fever in patients who eventually recover in about 5 to 9 days. In cases ending in death, clinical signs will occur at an early stage, and the patient will die between days 6 and 16 from hemorrhage and hypovolemic shock. The mortality rate is between 30% and 90% depending on the virus, with the highest rate reported for the Ebola-Zaire subtype. The Ebola-Reston subtype seems to possess a very low pathogenicity for humans or may be even apathogenic. Convalescence is prolonged and sometimes associated with myelitis, recurrent hepatitis, psychosis, or uveitis. An increased risk of abortion exists for pregnant women, and clinical observations indicate a high death rate for children of infected mothers *(229)*.

23.10.3.1 Treatment of Filovirus Hemorrhagic Fever

A filovirus-specific treatment does not exist *(229)*. Supportive therapy should be directed toward maintaining effective blood volume and electrolyte balance. The management of shock, cerebral edema, renal failure, coagulation disorders, and secondary bacterial infection is of ultimate importance for the survival of patients. Heparin treatment should only be considered when there is clear evidence of disseminated intravascular coagulopathy (DIC). Although human interferon and human reconvalescence plasma have been used in the past, there is a lack of experimental data showing efficacy. On the contrary, the filoviruses are resistant to the antiviral effects of interferon, and administering interferon to monkeys failed to increase the survival rate or to reduce the virus titer. Ribavirin is of no clinical value either.

Isolation of patients is strongly recommended, and the protection of medical and nursing staff is required.

23.10.3.2 Vaccines

Cross-protection among different Ebola subtypes in experimental animal models has been reported, thus suggesting a general value of vaccines. Inactivated vaccines have been developed by treating cell culture–propagated Marburg and Ebola, subtypes Sudan and Zaire, with formalin or heat. Protection, however, has only been achieved by careful balancing of the challenge dose and virulence. Immunizing monkeys with purified NP and GP has demonstrated the induction of the humoral and cellular immune responses and protection of animals against challenge with lethal doses *(229)*.

In a highly effective vaccine strategy for Ebola virus infection in cynomolgus macaques, a combination of DNA immunization and boosting with adenoviral vectors that encoded viral proteins generated cellular and humoral immunity *(230)*. Challenge with a lethal dose of the highly pathogenic, wild-type, 1976 Mayinga strain of Ebola-Zaire virus resulted in uniform infection in controls, who progressed to a moribund state and death in less than 1 week. In contrast, all vaccinated animals were asymptomatic for more than 6 months with no detectable virus after the initial challenge. These findings have demonstrated that it would be possible to develop a preventive vaccine against Ebola virus infection in non-human primates if the proposed vaccine strategy is proven effective against a more substantial viral challenge *(231–233)*.

In another study, monkeys have been protected against high doses of Marburg virus by a vaccine based on a modified Alphavirus construct *(234)*. The construct was an RNA replicon, based on the Venezuelan equine encephalitis (VEE) virus, which was used as a vaccine vector. The VEE structural genes were replaced by genes for the Marburg NP, GP, VP40, VP35, VP30, and VP24. Guinea pigs were vaccinated with recombinant VEE replicons (packaged into VEE-like particles) inoculated with the Marburg virus and evaluated for viremia and survival. The results indicated that either NP or GP were protective antigens, whereas VP35 afforded incomplete protection. In further evaluation of the vaccine's efficacy, cynomolgus macaques were inoculated with VEE replicons expressing the Marburg GP and/or NP. The results showed that the NP afforded incomplete protection, sufficient to prevent death but not disease in two of three macaques. Three monkeys vaccinated with replicons that expressed Marburg GP, and three others vaccinated with both replicons that expressed GP or NP, remained aviremic and were completely protected from disease *(234)*.

23.11 Viral Hemorrhagic Fever: Bunyaviridae

The family Bunyaviridae is one of the largest groupings of animal viruses comprising more than 300 species classified into five genera: Orthobunyavirus, Hantavirus, Phlebovirus, Nairovirus, and Tospovirus *(235)*. The Bunyaviridae have the capacity for sudden dramatic variation comparable with the antigenic shift associated with the influenza viruses, and this warrants continuing surveillance *(236)*. Several members of the Bunyaviridae family are the causative agents of the often lethal hemorrhagic fevers, most notably the Crimean-Congo hemorrhagic virus (Nairovirus), Rift Valley fever virus (Phlebovirus), Hantaan, Sin Nombre, and related viruses (Hantavirus).

Recently, another bunyavirus, Garissa (Orthobunyavirus) was found to be the cause of hemorrhagic fever *(237)*. The Garissa virus is now identified as an isolate of Ngari virus, which, in turn, is a Bunyamwera virus reassortant, which

acquired the L and S segments (near complete identity) of Bunyamwera virus *(238)*.

The Bunyaviridae virus particles are spherical, 90 to 100 nm in diameter and enveloped with glycoprotein surface projections. The virions contain three unique segments of negative-sense, single-stranded RNA in the form of circular ribonucleoprotein complexes (nucleocapsids) and a transcriptase enzyme. The nucleotide stretches are highly conserved at the segment ends *(236, 239–241)*. The 3′-terminal nucleotide sequences of the three genomic RNA segments are conserved within each genus.

Intrastrand base pair interaction between these terminal nucleotides leads to noncovalently closed, circular RNAs providing the functional promoter region for the interaction of the viral polymerase with the genome segments *(241)*.

The viruses replicate in the cytoplasm of the infected cell and mature by budding into smooth-surface vesicles in or near the Golgi region *(242)*. The budding site seems to be defined by the retention of the glycoproteins G_N and G_C at that particular site *(239, 242)*.

The Bunyaviridae viruses have the ability to interact genetically with certain other closely related viruses by *genome segment reassortment (238)*.

23.11.1 Crimean-Congo Hemorrhagic Fever Virus

Crimean-Congo hemorrhagic fever virus (CCHFV) is the causative agent of the Crimean-Congo hemorrhagic fever (CCHF). It is a member of the genus Nairovirus of the family Bunyaviridae *(239)*. The genus Nairovirus includes 34 identified viruses and is divided into seven different serogroups. Only three are known to be pathogenic to humans (CCHF, Dugbe, and the Nairobi sheep disease viruses) *(239)*.

23.11.1.1 CCHFV Structural Proteins

The three genome segments of the bunyaviruses encode four structural proteins: the RNA-dependent RNA polymerase (L protein) is encoded by the large (L) segment, which has recently been sequenced *(243, 244)*; the glycoproteins (G_N and G_C) are encoded by the medium (M) segment; and the nucleoprotein (N) is encoded by the small (S) segment *(239)*. Together with hantaviruses, the nairoviruses seem to possess the simplest genome expression strategy among the Bunyaviridae.

Structurally, CCHF virions represent spherical particles approximately 90 nm in diameter. The two glycoproteins G_N and G_C, which are inserted into the lipid envelope as spike-like structures, are responsible for the virion's attachment to receptors on the host cells and for the induction of neutralizing antibodies *(239)*.

The CCHFV glycoproteins are likely to play an important role in the natural tick-vertebrate cycle of the virus, as well as for the high pathogenicity in humans. Indeed, a highly variable mucin-like region at the amino terminus of the CCHFV glycoprotein precursor has been identified, a unique feature of nairoviruses within the family Bunyaviridae *(245)*. A similar serine-threonine–rich domain has been associated with increased vascular permeability and the development of hemorrhages in Ebola hemorrhagic fever *(218, 246)*.

The mature viral proteins, G_N and G_C, are generated by proteolytic cleavage from precursor proteins *(247)*. The amino termini of G_N and G_C are immediately preceded by the tetrapeptides RRLL and RKPL, respectively, leading to the hypothesis that SKI-1 or related proteases may be involved *(245)*. *In vitro* peptide cleavage data have shown that an RRLL tetrapeptide representing G_N processing site was efficiently cleaved by SKI-1 protease, whereas an RKPL tetrapeptide representing the G_C processing site was cleaved at negligible levels. The efficient cleavage of RRLL tetrapeptide is consistent with the known recognition sequences of SKI-1, including the sequence determinants involved in the cleavage of the Lassa virus (family Arenaviridae) glycoprotein precursor. Comparison of the SKI-1 cleavage efficiency between peptides representing Lassa virus GP2 and CCHFV G_N cleavage sites suggested that amino acids flanking the RRLL may modulate the efficiency. The apparent lack of SKI-1 cleavage of the CCHFV G_C RKPL site has indicated that related proteases other than SKI-1 are likely to be involved in the processing at this site and identical or similar sites used in several New World arenaviruses *(247)*.

After the complete genome sequence of the CCHFV reference strain IbAr10200 was determined, several expression plasmids were generated for the individual expression of the glycoproteins G_N and G_C, using CMV- and chicken β-actin-driven promoters *(248)*. The results of the study showed that the *N*-terminal glycoprotein G_N is localized in the Golgi complex, a process mediated by retention/targeting signal(s) in the cytoplasmic domain and ectodomain of this protein. In contrast, the *C*-terminal glycoprotein G_C remained in the endoplasmic reticulum but could be rescued into the Golgi complex by coexpression of G_N *(248)*.

23.11.1.2 Reverse Genetics

A thorough molecular analysis of CCHFV transcription/replication, protein biosynthesis, and processing would provide basic information to better understand the pathogenesis of the disease and to formulate concepts for antiviral treatment and vaccine development *(239)*. To this end, the generation of recombinant CCHFV particles based on DNA

transfection (infectious clone system) would greatly promote these studies.

As of recently, a new methodology, commonly referred to as *reverse genetics*, became available and allowed the genomes of negative-stranded viruses to be genetically manipulated and infectious viruses to be rescued entirely from cloned cDNAs. Application of reverse genetics has revolutionized the analysis of viral gene expression and has enabled the dissection of regulatory sequences important for replication and transcription *(249)*. The ability to rescue infectious viruses from cloned cDNAs has by now been well established for nonsegmented, negative-strand viruses, such as members of the families Rhabdoviridae *(250, 251)*, Paramyxoviridae *(252, 253)*, and Filoviridae *(254)*. However, the development of similar methodology for manipulating the genomes and generating viruses from cloned cDNAs of segmented, negative-strand viruses, such as members of the families Bunyaviridae, Orthomyxoviridae, and Arenaviridae, have turned out to be much more difficult *(249, 255)*.

Recently, a reverse genetics technology for CCHF virus was developed *(256)* using the RNA polymerase I system *(257, 258)*. Artificial viral RNA genome segments (minigenomes) were generated that contained different reporter genes in antisense (VRNA) or sense (cRNA) orientation flanked by the noncoding regions (NCR) of the CCHFV S segment. Reporter gene expression was observed in different eukaryotic cell lines after transfection and subsequent superinfection with CCHFV confirming encapsulation, transcription, and replication of the pol I–derived minigenomes. The successful transfer of reporter gene activity to secondary uninfected cells has demonstrated the generation of recombinant CCHFV and confirmed the packaging of the pol I–derived minigenomes into progeny viruses *(256)*. The system will offer a unique opportunity to study the biology of nairoviruses and to develop therapeutic and prophylactic measures against CCHFV infections *(239)*.

23.11.1.3 Genome Reassortment

One problem common to all segmented genome viruses is the production of virus particles that package the correct genetic complement *(236)*. In the Bunyaviridae viruses, the RNAs extracted from purified virus preparations are rarely equimolecular, and the S segment usually predominates. Although it is not clear how much of the deviation from an equimolar ratio is the result of degradation of the larger RNAs during the sample preparation, it does appear that virions containing different numbers of genome segments are produced *(259)*. A potential advantage of a segmented genome is the possibility for RNA segment reassortment to occur, which could confer new and desirable genetic traits on the progeny reassortants *(236)*.

In Bunyaviridae, reassortment has been demonstrated in the laboratory between certain related viruses, but not between viruses in different genera, nor between viruses in different serogroups in the same genus. There are further restrictions on reassortment, as seen by the difficulty in obtaining reassortants with a certain genotype (e.g., Batai, Bunyamwera, and Maguari bunyaviruses) where the L and S segments appeared to cosegregate in heterologous crosses, but this linkage could be broken when heterologous reassortants were used as parental viruses *(260)*. Genome segment reassortment has also been demonstrated experimentally in dually infected mosquitoes *(261–263)*, and evidence that reassortment also occurs in nature has been obtained *(237, 238, 264, 264a)*.

To assess the genetic reassortment of Rift Valley fever virus in nature, several isolates from diverse localities in Africa were studied by reverse transcription–PCR, followed by direct sequencing of a region of the small (S), medium (M), and large (L) genomic segments *(264b)*. The phylogenetic analysis showed the existence of three major lineages corresponding with geographic variants from West Africa, Egypt, and Central-East Africa. However, incongruences detected between the L, M, and S phylogenies suggested that genetic exchange through reassortment occurred between strains from different lineages. This hypothesis depicted by parallel phylogenies was further confirmed by statistical tests. The results of the study have strongly suggested that exchanges between strains from areas of endemicity in West and East Africa strengthen the potential existence of a sylvatic cycle in the tropical rainforest and also emphasize the risk of generating uncontrolled chimeric viruses by using live-attenuated vaccines in areas of endemicity *(264b)*.

23.11.2 Epidemiology of the Crimean-Congo Hemorrhagic Fever

The Crimean-Congo hemorrhagic fever is believed to be a very old disease, described as early as the 12th century, from southeastern Russia in the region that is currently Tadzhikistan *(264c)*. The first well-documented report of CCHF was from an epidemic in the western Crimea region of Russia (currently in Ukraine) *(264d)*. Later, a similar disease was observed in Africa in 1956 *(265)*; the virus was subsequently isolated and found to be antigenically indistinguishable from the Crimean hemorrhagic fever virus *(266)*. The name Crimean-Congo hemorrhagic fever virus was suggested and commonly accepted to designate the disease *(267)*.

The known distribution of the CCHFV covers the greatest geographic range of any tick-borne virus, with reports of virus isolation and/or disease from more than 30 countries

in Africa, Asia, southeastern Europe, and the Middle East *(239)*.

In general, the known occurrence of CCHFV in Europe, Asia, and Africa coincides with the world distribution of ticks of the genus *Hyalomma* (e.g., *Hyalomma marginatum*) of the Ixodidae family *(264c)*, followed by members of the genera *Rhipicephalus* and *Dermacentor*. Altogether, the virus has been isolated from at least 31 species of ticks and one species of biting midge (*Culicoides* spp.).

The natural cycle of CCHFV includes transovarial (i.e., passed through the eggs) and transstadial (i.e., passed directly from immature ticks to subsequent life stages) transmission among ticks in a tick-vertebrate-tick cycle involving a variety of wild and domestic animals. *Hyalomma* ticks normally feed on livestock (sheep, goats, and cattle), large wild herbivores, hares, and hedgehogs, which become infected with CCHFV *(264c)*. Infection in these animals generally results in an unapparent or subclinical disease but generates viremia levels capable of supporting virus transmission to uninfected ticks *(264c)*. Regarding the role of birds in the epidemiology of CCHF, it appears that birds (with the exception of ostriches) are refractory to CCHFV infection, even though they can support large numbers of CCHFV-infected ticks.

Transmission to humans can occur either through tick bites or possibly by crushing engorged infected ticks. Direct contact with virus-contaminated blood or tissues from infected animals or humans is another source of virus transmission and is generally characterized by more severe clinical symptoms and high mortality *(239, 268)*.

23.11.3 Crimean-Congo Hemorrhagic Fever: Clinical Manifestations

The family Bunyaviridae contains at least 41 tropical viruses that cause fever *(239)*. Some of these viruses also cause meningoencephalitis, hemorrhage, arthritis, or retinitis.

The CCHFV is associated with hemorrhages and is often fatal *(239,264c,269)*. Outbreaks of CCHF are focal in nature, and clinically they present with fever, malaise, and prostration, to severe and fatal hemorrhagic disease. The fatality rate varies from 25% to more than 75%, with most deaths occurring 5 to 14 days after the onset of illness.

Four different phases of the disease are recognized: incubation; prehemorrhagic; hemorrhagic; and convalescence.

The incubation period of the CCHFV is between 3 and 6 days in nosocomial outbreaks and 2 to 12 days in other situations *(264c)*.

The prehemorrhagic phase is characterized by a sudden onset of flu-like symptoms, including dizziness, neck pain and stiffness, myalgia, back and abdominal pain, severe headache, eye pain and photophobia, arthralgias, chills, anorexia, sore throat, nausea, and vomiting. Neuropsychiatric changes have also been reported—patients have developed altered consciousness progressing to aggressive behavior and unconsciousness *(270)*. About 50% of the patients will develop hepatomegaly.

In moderate to severe illness, fever will last between 5 and 20 days (average, 9 days). The disease may be biphasic, with fever followed by two afebrile days, then a return of fever associated with epistaxis, petechiae, purpura, and thrombocytopenia *(239)*.

Hemorrhage often begins with blood leakage at sites of needlesticks and may become quite profuse. Shock ensues in severe cases and may be accompanied by liver failure, cerebral hemorrhage, anemia, dehydration, diarrhea, myocardial infarction, pulmonary edema, and pleural effusions *(239)*.

The convalescence phase starts 15 to 20 days after the onset of illness and is characterized by weakness, weak pulse, confusion, asthenia, alopecia, and neuralgias for 2 to 4 months *(264c)*.

Factors contributing to a fatal outcome include hemorrhage, severe dehydration, severe anemia, and shock associated with prolonged diarrhea, lung edema, myocardial infarction, and pleural effusion. Multiple organ failure is common, and liver lesions may vary from disseminated necrotic foci to massive necrosis *(218)*.

23.11.3.1 CCHF: Diagnosis, Treatment, and Prognosis

Bunyaviral fevers are commonly encountered in the tropics. Even though an early diagnosis of CCHF is very important for the disease outcome in the patient, the cause is usually left undetermined because laboratory diagnostic capabilities are lacking *(239)*. The differential diagnosis should include borreliosis, leptospirosis, and rickettsiosis. Usually the first indicators of CCHF are the clinical symptoms combined with the patient's history (e.g., tick bites, travel to endemic areas, and exposure to blood or tissue of livestock or human patients).

From the available classic and modern techniques that can be used to diagnose CCHF, the antigen capture enzyme-linked immunoassay (ELISA) seems to be the most efficient, allowing viral antigen in the patient's serum to be detected in 5 to 6 hours; however, this assay is less sensitive *(239)*. Nevertheless, ELISA is of value for the more severe CCHF cases because of higher viral titer and prolonged viremia. Recently, new immunologic assays have been reported using recombinant CCHFV nucleoprotein in an indirect fluorescent-antibody (IFA) assay as well as an ELISA assay *(271, 272)*.

Treatment options for CCHF are very limited. Currently, there is no antiviral therapy available for use in humans with CCHF. Supportive therapy still remains the major means of controlling hematologic and fluid imbalances *(239)*.

Ribavirin, an antiviral nucleoside analogue, has been found effective for inhibiting CCHFV replication *in vitro (273)* and in animal models *(274)*. Case reports have suggested that oral or intravenous administration of ribavirin has been helpful in treating human CCHF *(275–278)*. However, there are no randomized, controlled clinical studies to confirm these reports. Suggested doses for intravenous ribavirin are 30 mg/kg for one dose, then 16 mg/kg every 6 hours for 4 days; then 8 mg/kg every 8 hours for 6 days *(279)*.

Immunotherapy using passive transfer of CCHF survivor plasma (hyperimmune serum) has also been used *(280)*. Although limited in scope, these studies have suggested that the CCHF immune serum may be beneficial when administered intravenously at a dose of 250 mL over 1 to 2 hours on successive days and when given early in the infection *(280, 281)*. The beneficial effect of this treatment has been based on observed clinical improvement, but the study did not include placebo control patients.

A vaccine for CCHF derived from inactivated mouse brain has been in use in Bulgaria *(282, 283)* but is not available outside of the country. It is not clear whether the efficacy of this vaccine has been well quantified.

23.11.4 Rift Valley Fever Virus

Rift Valley fever is considered to be one of the most important viral zoonoses in Africa, and the causative viral agent was first isolated in 1931 after an outbreak with large number of aborting sheep and lamb deaths near Naivasha in the Rift Valley of Kenya. At the same time, it was noticed that people attending to the sheep simultaneously suffered from a febrile illness *(284)*. The Rift Valley fever virus (RVFV) was classified later as member of the Bunyaviridae family, genus Phlebovirus. Initially, the virus was endemic to the region, but later spread to West, Central, and southern Africa and Madagascar. In 1997, RVFV provoked a sudden and dramatic outbreak in Egypt, then in 2000 RVFV spread outside Africa to the Arabian Peninsula and caused two simultaneous outbreaks, in Yemen and Saudi Arabia *(284)*. Phylogenetic analysis indicated three major lineages for RVFV: Western Africa, Eastern/Central Africa, and Egypt. All of the strains isolated from the Arabian Peninsula and Madagascar were closely related to each other and mapped together with the Kenyan isolate. The phylogenetic studies also revealed the ability of this segmented RNA virus to exchange genetic material between isolates from different geographic areas *(264b)* (see Section 23.11.1.3).

RVHV is transmitted to ruminants and humans by the bite of infected mosquitoes. Occasionally, humans may be infected by contact with infected tissues or aerosols. Circumstantial evidence exists that infection can occur through contact with raw milk, although very rarely *(285)*.

In Africa, 23 species of mosquitoes from the genera *Aedes*, *Culex*, *Anopheles*, *Eretmapodites*, and *Mansonia* have been found to be involved in RVFV transmission. In addition, numerous strains of RVFV have been isolated from various species of biting midges (*Culicoides*), black flies (*Simuli*), and occasionally from ticks (*Rhipicephalus*). In some cases, the virus can also be transmitted mechanically *(284)*.

23.11.4.1 RVFV: Molecular Biology

Like all of the Bunyaviridae, RFFV has a tripartite single-stranded RNA genome consisting of L (large), M (medium), and S (small) segments *(284)*. The L and M segments are of negative polarity and express, respectively, the RNA-dependent RNA polymerase L and the precursor to the glycoproteins G_N and G_C. Posttranslational cleavage of this precursor protein also generates a nonstructural protein (MSm) of yet undetermined role.

The S segment of RVFV (like all other phleboviruses) uses an ambisense strategy and encodes for the nucleoprotein N in antisense and for the nonstructural protein NSs in sense orientation.

The viral genes are flanked by noncoding regions containing important *cis*-acting elements for the regulation of viral genome transcription, replication, encapsulation, and packaging into progeny virions *(284)*.

A highly efficient reverse genetics system was developed that allowed generation of recombinant RVFVs to assess the role of NSm protein in virulence in a rat model in which wild-type RVFV strain ZH501 (wt-ZH501) resulted in 100% lethal hepatic disease 2 to 3 days after infection *(286)*.

Minigenome Systems

Recently, it has become possible to genetically manipulate the genomes of negative-stranded viruses and to generate infectious virions entirely from cloned cDNAs. By using *minigenome rescue systems*, this reverse genetics technique has revolutionized the study of viral gene expression and has enabled regulatory elements required for viral transcription and replication steps to be dissected, as well identifying viral components interacting with the host cells *(249–255)* (see Section 23.11.1.2).

In a typical minigenome system, virus-like RNA (minigenome) transcripts contain an internal open reading frame (ORF) of a reporter gene in place of a viral ORF sandwiched by untranslated regions (UTRs) of viral RNA termini. The expressed minigenome RNA transcripts undergo RNA replication and transcription in the presence

of coexpressed viral proteins or co-infected helper virus; the levels of reporter expression are a measure of the efficiency of minigenome RNA replication and transcription. Minigenome transcripts are expressed by either host RNA polymerase I or T7 RNA polymerase, the latter of which is often provided by vaccinia virus or Sindbis virus *(287)*.

However, in the case of RVFV, the reverse genetics system was relatively inefficient and it was not possible to clearly demonstrate whether replication did occur. For this reason, an RNA polymerase I (pol I)-driven system *(287, 288)* has been developed and found to be more efficient and functional for transcription and replication *(284, 289)*. A minigenome rescue system expressing a CAT reporter has been established to investigate the role of the noncoding regions in this process. The results of the study have shown that the L, M, and S segment-based minigenomes have driven *bona fide* transcription and replication and have expressed variable levels of CAT reporter, indicating differential promoter activities within the noncoding sequences. In addition, there was a good correlation between the relative promoter strength and the abundance of viral RNA species in RVFV-infected cells *(290)*.

The coexpression of NSs protein with L and N proteins substantially enhanced minigenome replication and transcription, suggesting that RVFV NSs protein played a critical role in the RVFV RNA synthesis *(287)*. The enhancement of the minigenome RNA synthesis by the NSs protein occurred in cells lacking α/β interferon (IFN-α/β) genes, indicating that the effect of NSs protein on minigenome RNA replication was unrelated to a putative NSs protein–induced inhibition of IFN-α/β production *(287)*. Unlike other viral IFN antagonists, NSs did not inhibit IFN-specific transcription factors but blocked the IFN gene expression at a subsequent step *(291)*.

The Interferon Response Circuit

The type I interferons (IFN-α/β) are potent antiviral cytokines and modulators of the adaptive immune system. They are induced by viral infection or by double-stranded RNA (dsRNA), a by-product of viral replication, and lead to the production of a broad range of antiviral proteins and immunoactive cytokines. However, viruses, in turn, have evolved multiple strategies or mechanisms to counter the IFN system, which would otherwise stop the virus growth early in infection, leading to a complicated balancing act between the virus-induced IFN responses and the viral escape countermeasures by suppressing IFN production, down-modulating IFN signaling, and blocking the action of antiviral effector proteins *(292)*.

Induction of type I (α/β) IFN gene expression is tightly regulated. Recent findings suggested that the cells make use of two major but distinct cellular signal transduction pathways to sense viruses and activate their type I IFN genes. Most cells in the body, including fibroblasts, hepatocytes, and conventional dendritic cells, use the *classic pathway*. Using their intracellular sensors, upon infection these cells will detect viral components in the cytoplasm and then activate the main IFN regulatory transcription factors IRF-3 and NF-κB, which in turn will transactivate the IFN-β gene expression. Infected cells secrete mainly IFN-β as an initial response to infection but would switch to IFN-α during the subsequent amplification phase of the IFN response *(293)*. In contrast, plasmacytoid dendritic cells use Toll-like receptors expressed on the cell surface or in endosomes to sense extracellular or engulfed viral material.

The IFN receptor signaling pathway is now firmly established, and IFN-β and multiple IFN-α subspecies activate a common type I IFN receptor (IFNAR), which sends a signal to the nucleus through the so-called JAK-STAT pathway *(292, 294)*.

Type I IFNs activate the expression of several hundred IFN-stimulated genes *(295)*, some of which code for antiviral proteins via three antiviral pathways: the protein kinase R, the 2,5-OAS/RNasel system, and the Mx proteins *(292)*.

23.11.4.2 Pathogenesis and Immune Response

The RVFV affects primarily the liver, with rapid hepatocellular changes progressing to massive necrosis. Hepatic necrosis and vasculitis are the most characteristic microscopic lesions of RVFV in domestic animals and humans *(284)*. A profound leukopenia, elevated serum enzymes associated with severe liver damage, and thrombocytopenia are strongly associated with the development of the hemorrhagic state *(296)*. It is likely that the virus is transported from the inoculation site to the lymph nodes by lymphatic drainage. Once in the lymph nodes, the virus replicates and is spread into the circulation to produce primary viremia, leading to infection of the target organs *(284)*. In addition, RVFV replicates in hepatocytes in the liver and in the walls of small vessels (adrenocortical cell and glomeruli of the kidney).

As with most viral infections, RVFV is expected to induce both adaptive and innate immune responses *(284)*. It is a common feature of bunyavirus infections that the antibody response plays an important role in protection. Antibodies are raised against the internal nucleoprotein (the major immunogen) and the surface glucoproteins G_N and G_C, which carry neutralizing epitopes *(297)*.

With respect to innate immunity, major studies have been carried out on the virulence of the nonstructural protein NSs, which antagonizes the antiviral response by blocking type I interferon production *(298)*. As a general inhibitor of transcription, NSs must have a wide range of action on cellular

transcription by not only inhibiting the antiviral response and preventing the synthesis of IFN-β but also by affecting the transcriptional activity in response to hormonal stimuli *(284)*.

Infection with RVFV leads to a rapid and drastic suppression of host cellular RNA synthesis that parallels a decrease in the transcription factor II H (TFIIH) cellular concentration *(299)*. It was further found that the nonstructural viral protein NSs interacts with the p44 component of TFIIH to form nuclear filamentous structures that also contain the XPB subunit of TFIIH. By competing with XPD (the natural partner of p44 within TFIIH), and sequestering the p44 and XPB subunits, NSs prevented the assembly of the TFIIH subunits, thus destabilizing the normal host cell life *(299)*. These observations shed light on the mechanism used by the RVFV to evade the host response.

23.11.4.3 Clinical Manifestations

The incubation period of the disease varies from 2 to 6 days. The symptoms include an influenza-like illness, headache, myalgia, and backache. Some patients will present with neck stiffness, photophobia, and vomiting. The symptoms of Rift Valley fever (RVF) usually last from 4 to 7 days, after which time the immune response becomes detectable with the appearance of IgM and IgG antibodies, and the disappearance of circulating virus from the bloodstream (http://www.who.int/mediacente/factsheets/fs207/en).

Whereas most human cases are relatively mild, a small proportion of patients (0.5% to 2%) may develop a much more severe illness characterized with several recognizable syndromes: eye disease (retinal lesions), meningoencephalitis, or hemorrhagic fever (severe liver disease, jaundice, vomiting blood, passing blood in the feces, purpuric rash, and bleeding from the gums). The case-fatality rate from hemorrhagic disease is about 50% (http://www.who.int/mediacente/factsheets/fs207/en).

23.11.4.4 Treatment

Because most human cases of RVF are relatively mild and of short duration, they will not require any specific treatment.

Concerning treatment, it has been shown that administrations of antibodies, interferon, interferon inducer, or ribavirin in experimentally RVFV-infected mice, rats, and monkeys have exerted protective effects against the disease *(300)*. However, these treatments have never been tested to treat RVFV infection in humans.

An inactivated vaccine has been developed for human use. Although the vaccine has been used to protect veterinary and laboratory personnel at high risk of exposure to RVF, it is not licensed and not available commercially. Other candidate vaccines are under investigation.

23.11.5 Hantaviruses

Hantaviruses are trisegmented, negative-strand RNA viruses that belong to the family Bunyaviridae *(301)*. Unlike other viruses of the same family, in nature, the hantaviruses do not have an arthropod vector but are exclusively maintained in the population of their specific rodent hosts *(302)*. In their natural host species, hantaviruses usually develop a persistent infection with prolonged virus shedding in excreta. Humans become infected by inhaling virus-contaminated aerosol, but unlike the asymptomatic infection in rodents, in humans the hantaviruses are the etiologic agents of two acute febrile diseases: hemorrhagic fever with renal syndrome (HFRS) and hantavirus pulmonary syndrome (HPS) *(303, 304)*. The mortality rate varies from 0.1% to 40% depending on the virus involved.

Both HFRS and HPS appear to be immunopathologic, and inflammatory mediators are important in causing the clinical manifestations. In HPS, T cells act on heavily infected pulmonary endothelium, and both IFN-γ and TFN-α are major agents of a reversible increase in vascular permeability that leads to severe, noncardiogenic pulmonary edema. HFRS has prominent systemic manifestations in which the retroperitoneum is a major site of vascular leak and the kidneys suffer tubular necrosis. Both syndromes are accompanied by myocardial depression and hypotension or shock *(304)*.

While HFRS is primarily a Eurasian disease, HPS appears to be confined to the Americas. These geographic distinctions correlate with the phylogenies of the rodent hosts and the viruses that co-evolved with them.

23.11.5.1 Hantavirus Genome

The Hantavirus genome consists of large (L), medium (M), and small (S) segments that code for the viral RNA-dependent RNA polymerase (RdRp), the glycoprotein precursor (GPC) of two envelope glycoproteins (G1 and G2), and the nucleocapsid protein (N), respectively. In the virion, the viral RNA is complexed with the multiple N protein molecules to form individual L, M, and S ribonucleocapsid structures. Furthermore, the nucleocapsids and RdRp are packaged within a lipid envelope that is embedded with viral glycoproteins. Each virion usually contains equimolar amounts of the three genomic viral RNAs *(305)*.

Nucleocapsid Protein

The nucleocapsid (N) protein is the most abundant Hantavirus protein synthesized early after infection, and similarly to other Bunyaviridae viruses, it is central to virus replication. It may also protect newly synthesized viral RNA (vRNA) from nuclease degradation as well as play a role in

preventing intrastrand base pairing within the vRNA template *(301)*. The N protein binds to membranes and vRNAs, associates with transcription and replication complexes, and oligomerizes during the process of virus assembly. The pathway of Hantavirus nucleocapsid oligomerization involves helix bundling or alternative amino-terminal coiled-coil conformation *(306)*.

In addition, recent studies provided evidence that the N protein can interact with host cell proteins. Although the nature of this interaction is unclear, it may be hypothesized that it is important for virus replication by blocking the inhibitory action of the host cell antiviral proteins and preventing infected cell apoptosis *(301)*.

Glycoproteins

The Hantavirus glycoproteins precursor (GPC) is synthesized in the ribosomes bound to the endoplasmic reticulum (ER) *(307)*. The GPC is then translocated into the ER lumen and is co-translationally cleaved. Two glycoproteins (G1 and G2) are generated by posttranslational cleavage of GPC at the conserved amino acid sequence WAASA *(308)*, and G1 and G2 are glycosylated to form heterodimers that constitute a Golgi retention signal. However, for Golgi translocation of the glycoproteins, they have to be coexpressed *(308)*, resulting in the formation of a G1/G2 complex. In the Golgi, G1/G2 complexes complete maturation and become ready for virus assembly *(309)*.

The G1 and G2 glycoproteins are known to mediate cell attachment and fusion *(310–312)*. It has been demonstrated that the hantaviruses use integrin receptors for cell attachment. Thus, β_3 integrins, which regulate vascular permeability and platelet function and are present on the surfaces of platelets, endothelial cells, and macrophages, facilitate the cellular entry of HFRS-associated hantaviruses *(313)*.

Viral RNA–Dependent RNA Polymerase (RdRp)

Hantavirus RNA-dependent RNA polymerase (RdRp) mediates transcription and replication. Therefore, it has been suggested that RdRp should have endonuclease, transcriptase, replicase, and possibly RNA-helicase activities *(301)*. Shortly after virion entry, RdRp initiates transcription and uncoating *(314)*.

23.11.5.2 Hantaviruses and Their Diseases

It is well known that, in general, the geographic distribution and epidemiology of hantaviruses reflect the distribution and natural history of their primary rodent hosts. To this end, there are several distinct, regional hantaviruses:

(i) Hantaan virus or Korean hemorrhagic virus (also known as epidemic hemorrhagic fever), found in Asia; etiologic agent of HFRS.
(ii) Seoul virus, associated with domestic rats, and causing HFRS.
(iii) Sin Nombre virus (previously known as Muerto canyon virus and Four Corners virus), found in the United States, and causing Hantavirus-associated respiratory/pulmonary syndrome (HARDS).
(iv) Puumala virus, found in Scandinavia, Europe, and western Russia.
(v) Dobrava or Belgrade virus, found in Eastern Europe; etiologic agent of HPS.
(vi) Baltimore rat virus or New York virus, a fairly unrecognized form that is suspected of causing chronic renal disease and/or hypertension, as well as HPS.

In addition, other serologically distinct viruses causing human disease have been identified, including [*Note: human disease and geographic distribution given in parenthesis*]: Amur virus (HFRS; Siberia, Far East, Russia), Dobrava-like virus (HFRS; Europe, Northern Caucasus), Andes virus (HPS; South America: Argentina and Chile), Lechiguanas virus (HPS; South America: Argentina), Oran virus or "Andes-Nort" virus (HPS; South America), Laguna Negra virus (HPS; South America), Choclo virus (HPS; South America), and Araucaria virus (HPS; South America: Brazil).

Although hantavirus diseases are associated with seasonal changes that coincide with the life cycle of the host rodents, the greater incidence of HFRS cases occur in rural areas among farmers, forest workers, military personnel, and so forth, and always coincides with increased human agricultural activities in spring and autumn.

No vectors have been shown to be involved in the Hantavirus transmission cycle. It is believed that infection is spread horizontally by inhaling virus-contaminated aerosol, and very rarely through bites. Infectious virus is shed in the rodent's saliva, urine, and feces, possibly throughout the rest of its life.

With the exception of the Andes virus, there is no evidence of human-to-human transmission of Hantavirus. In fact, human-to-human transmission has not been confirmed with the Andes virus, but the pattern of infection implied a human vector (http://www.cdc.gov/ncidod/EID/vol11no12/05-0501.htm).

Some of the known rodent vectors associated with human disease include *Apodemus agrarius*—Asian subspecies (Hantaan virus), *A. flavicollis* and *A. agrarius* (Dobrava virus), *A. ponticus* (Dobrava-like virus), *Rattus norvegicus* (Seoul virus), *Clethrionomys glareolus* (Puumala

virus), *Peromyscus maniculatus*—grassland subspecies (Sin Nombre virus), *P. maniculatus*—forest species (Monongahela virus), *Oligoryzomys longicaudatus* (Argentina), and *Calomys laucha* (Choclo virus).

Genome segment reassortment is one of the important ways used by segmented viruses to achieve high infectivity and adaptation to new animal species *(315–317)*. Thus, preferential reassortment with homologous L-M and L-S segments has been described for many viruses of the Bunyaviridae family *(318–320)*.

23.11.5.3 Pathogenesis and Immune Responses to Hantavirus Diseases

Currently, there is no single factor identified to explain the complexity of the pathogenesis of HFRS and HPS *(301)*. Most likely, the Hantavirus pathogenesis is a multifunctional process involving mainly endothelial cell damage, immune effectors, cytokines, and chemokines *(321, 322)*.

Immune complexes (ICs) were found in the HFRS serum before Hantavirus-specific antibodies could be detected. The levels of ICs gradually increase during the febrile, oliguric, and polyuric phases of HFRS, and then usually decline during the convalescent phase of the disease. However, it is still not clear whether the levels of serum ICs correlate with the severity of clinical manifestations *(323, 324)*. Furthermore, electron microscopic studies of kidney biopsies collected 3 days after the onset of HFRS revealed C3 complement component and IgM deposits along the basal capillary membrane of the glomeruli, and in the interstitial tissue of kidney later during HFRS *(325, 326)*. The morphologic hallmarks of ICs-mediated tissue injury are necrosis and predominately neutrophil-composed cellular infiltrates.

Classic and alternative pathways of complement activation are induced in HFRS patients, and the severity of the disease correlates with the degree of complement activation *(301)*. Increased serum levels of activated C1 complement component were found in HFRS patients 5 to 6 days after the onset of disease, and its presence coincides with manifestations of the hemorrhagic syndrome, proteinuria, and shock *(327)*. A number of studies have suggested that the nature of ICs formed during HFRS (in particular, ICs containing IgG1 and IgG3 subclass antibody) may contribute to complement activation and prolonged ICs circulation in the blood *(301)*.

B-Cell Immune Responses

HFRS and HPS patients have manifested with high levels of Hantavirus IgM detected simultaneously with the onset of clinical symptoms. The IgM antibodies were directed against all three of the structural proteins of Hantavirus *(328)*. The IgM levels declined during the convalescent stage of the disease, which usually coincides with the rise of Hantavirus IgG *(301)*.

In general, the IgG subclass responses are comparable for both HFRS and HPS patients. The Hantavirus induces an early IgG1 response that increases with the progression of the disease, with antibody titers higher against the G1 and G2 glycoproteins than against the N protein *(301)*.

The neutralizing IgA response is most important in the development of mucosal immunity, especially in recovery from acute infection and long-term immunity. The mechanism of antiviral IgA protection remains unclear, although some evidence suggests that it may interfere with the replication of the virus by binding to newly synthesized viral proteins within infected cells and neutralizing the virus intracellularly *(329)*.

Hantavirus infection has been shown to significantly increase the serum levels of IgE compared with that of other viral diseases, such as influenza, cytomegalovirus, and Epstein-Barr virus *(330)*.

T-Cell Responses

Detection of virus strain–specific cytotoxic T-lymphocytes (CTL) in the blood of convalescent donors suggests that CTL plays an important role in the pathogenesis of hantaviruses. The Hantavirus N protein is believed to be the major antigen activating the CTL response *(331, 332)*. Although the importance of virus-specific $CD8^+$ CTL for clearance of the virus and recovery is well recognized, virus clearance by the $CD8^+$ CTL has often been associated with apoptosis of infected cells and tissue damage. In fact, viral clearance and tissue injury (destruction of infected endothelial cells) are believed to be two interrelated consequences of the cellular immune response *(332, 333)*.

CTL can control virus infections and induce disease pathogenesis by secreting cytokines such as IFN-γ and TFN-α *(334, 335)*. Cytokines play a major role in facilitating the $CD8^+$ CTL cytotoxic activity, and the cytokine profile [as a function of the particular viral epitope *(336)*] may contribute to the lung injury. Furthermore, proinflammatory cytokines secreted by activated $CD8^+$ T-cells can attract inflammatory cells to the site of infection and activate parenchymal cells to release more chemokines, such as IP-10 and Mig *(337)*, and monocyte chemotactic protein-1 (MCP-1) and the macrophage inflammatory protein-2 *(338)*.

23.11.5.4 Hantavirus Hemorrhagic Fever with Renal Syndrome

Epidemiology

HFRS (also known as the *Korean hemorrhagic fever*, epidemic nephrosonephritis, nephropathica epidemica, Hantaan

fever, Hantaan hemorrhagic fever, and Songo fever) caused by the hantaviruses is a major public health problem, with hundred of thousands of cases annually in Asia (China, Korea, and eastern Russia) and Europe (Scandinavia, western Europe, the Balkans, and western Russia).

The Korean hemorrhagic fever, caused by a Hantavirus infection, first attracted attention during and after the Korean War (1951–1953), when more than 3,000 U.S. and Korean soldiers fell severely ill with an infectious disease characterized by renal failure, generalized hemorrhage, and shock, and with a mortality rate over 10%. The causative agent was first isolated in 1976 from the lungs of the striped field mouse, *Apodemus agrarius*, and designated as the Hantaan virus *(339, 340)*.

The incidence of HFRS in the United States is not very well known. The most likely etiologic agent is the Seoul virus (vector: *Rattus norvegicus*), which causes mild to moderately severe HFRS in Asia and is the only Hantavirus associated with urban disease *(303, 304)*. The rodent and its virus have been spread around the world by maritime commerce. The prevalence of antibodies in inner city residences in the United States has been estimated to be about 1% or less, with only several acute cases suggestive of Seoul virus being reported *(341)*. Other North American hantaviruses from voles (e.g., *Microtus pennsylvanicus*) have not yet been implicated as causes of HFRS *(303, 304)*.

Clinical Manifestations of HFRS

Symptoms of HFRS usually occur between 1 and 6 weeks after exposure to the virus. The initial onset is marked by nonspecific flu-like symptoms: fever, myalgia, headache, abdominal pain, nausea, and vomiting. There is a characteristic facial flushing, and usually a petechial rash (mainly limited to the axilla or the armpits). Sudden and extreme albuminuria will occur about day 4; this is characteristic of severe HFRS. Ecchymosis also occurs, and commonly, scleral injection and bloodshot eyes. Additional symptoms may include hypotension, shock, respiratory distress and/or failure, and renal impairment and/or failure. *Damage to the renal medulla is characteristic and unique for the hantaviruses*.

There are five phases of HFRS, although any one or more of these stages may not be apparent *(304)*:

(i) *Febrile*—lasting 3 to 5 days; it may often include the initial symptoms of the disease: thirst, nausea and vomiting, abdominal pain, blurred vision and photophobia, characteristic flushing of the face and the V-area of the neck and thorax, conjunctival suffusion, and periorbital edema.

(ii) *Hypotensive*—lasting for a few hours to 2 days; nausea and vomiting are common, thrombocytopenia is almost universal, and laboratory evidence of disseminated intravascular coagulation is common. The blood pressure then falls, perhaps with severe or fatal shock. One-third of the deaths will occur at this stage.

(iii) *Oliguric*—lasting from few days to 2 weeks; marked polymorphonuclear leukocytosis with a left shift is typically present, and $CD8^{+}$-activated T cells appear on the peripheral blood smear as atypical lymphocytes. Proteinuria is marked and the urine-specific gravity falls to 1.010, followed by oliguria. CNS manifestations (obtundation or coma) may be present either from the disease process or from metabolic disturbances. Hemorrhagic tendencies continue, including ecchymoses and mucous membrane hemorrhages. Half of all deaths occur during this stage.

(iv) *Polyuric/diuretic*—renal function returns within a few days to 2 weeks but the subsequent polyuria and hyposthenuria pose new problems in fluid and electrolyte management. Death can still occur from shock or pulmonary complications.

(v) *Convalescent*—fluid and electrolyte imbalance still exist and can last weeks or months.

The more severe cases of HFRS are usually caused by the Hantaan virus infections in Asia or Dobrava virus infection in the Balkans *(341a, 341b)*, both of which have a case fatality rate of 5–15%. The milder cases of HFRS (caused generally by the Seoul or Puumala viruses) usually do not display the full spectrum of clinical manifestations and have a case fatality of less than 1% *(304)*.

Management of HFRS

Successful treatment of HFRS begins with prompt diagnosis. Shock is usually managed with the administration of pressors and judicious administration of fluids—1 to 2 fluid units of human serum albumin is thought to be a useful adjunct *(304)*. Dialysis reduces the mortality rate from 5% to 15% to less than 5% and should begin promptly to treat hyperkalemia, volume overexpression, or uremia. Furthermore, volume adjustment is particularly important because pulmonary edema and intracerebral hemorrhage are two major causes of death in the oliguric phase of the disease. Later, diuresis of large volumes of isosthenuric urine will lead to potentially fatal volume and electrolyte abnormalities *(304)*.

Intravenous ribavirin, when given within the first 4 days of onset, has been shown to lessen renal failure, decrease the bleeding manifestations, and decrease the case fatality rate in a Chinese setting in which dialysis and other supportive measures were less available *(342, 343)*.

The classic Salk-type Hantavirus vaccines have been widely and successfully used for a number of years in Korea and China. Meanwhile, more sophisticated recombinant and DNA vaccines are being developed in Europe and North America, but none has entered the market *(344)*. In addition to cloning sequences for Hantavirus genomes for diagnostic purposes, researchers at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) have constructed and engineered a vaccine for Hantaan virus that expresses the virus M gene products, G1 and G2 *(344a)*. Other vaccine developments include testing of suckling mouse and suckling rat brain vaccines in North and South Korea *(345)*, as well as other inactivated vaccines *(346)*.

23.11.5.5 Hantavirus Pulmonary Syndrome

Epidemiology

The initial association of HPS was with the Sin Nombre virus and its rodent reservoir, the deer mouse (*Peromyscus maniculatus*). Later, HPS cases were identified outside the range of the deer mouse, and the Bayou, Black Creek Canal, and the New York viruses have been implicated as the etiologic agents of HPS; each apparently infects a distinctive species of rodents *(304)*. Although the number of these recognized HPS cases is small, there may be more prominent component of renal failure in the Bayou and Black Creek Canal infections *(347–350)*.

Studies in South America have also identified an increasing number of viruses that cause HPS in humans, but only chronic asymptomatic infections in rodents of the Sigmodontinae subfamily of the family Muridae *(351)*. The actual incidence of HPS in South America is not known, but local epidemics and sporadic cases have been reported in several countries *(304)*.

Human-to-human transmission has not been recognized in any Hantavirus disease, with exception of a single HPS epidemic caused by the Andes virus in Argentina in 1996 *(352, 353)*. A combination of human epidemiology, rodent studies, and molecular genetics of the virus has established the transmission.

Clinical Manifestations of HPS

The HPS is primarily a lung infection, with the kidneys largely unaffected. The initial manifestations of HPS include mainly flu-like symptoms: fever, myalgia, headache, and cough; other symptoms can include chills, abdominal pain, diarrhea, and malaise. However, cardiopulmonary dysfunction, ranging from mild hypoxemia with stable hemodynamics to rapidly progressive respiratory failure with cardiogenic shock, is the hallmark of patients with fully developed HPS *(304, 351)*. The disease progresses rapidly. Eventually, the patients experience thrombocytopenia, hypoxemia, and interstitial pulmonary edema concurrently with hypotension, shock, and respiratory distress followed by respiratory failure. Most patients infected with Sin Nombre virus will die within few days of onset of symptoms.

Patients with severe pulmonary edema have copious, amber-colored, nonpurulent pulmonary secretions associated with diffuse pulmonary infiltrates on chest radiograph.

The average duration of HPS prior to hospitalization is 3 to 4 days, and in those patients who deteriorated, the usual time until death was an additional 1 to 3 days, although patients can die within hours of admission. Severe cardiopulmonary dysfunction predicts a poor prognosis in HPS, with myocardial depression leading to shock and severe oxygen deficiency dominating the hemodynamic picture *(304)*.

There has been a general absence of associated multiple organ failure, renal failure, or hepatic failure in Sin Nombre virus infections.

Management of HPS

During diagnosis, HPS is commonly confused with acute respiratory distress syndrome from other infectious pathogens, pyelonephritis, intra-abdominal processes, pneumonias, and systemic infections such as rickettsial disease or plague. Therefore, early diagnosis for optimal management is paramount.

No instance of person-to-person transmission of HPS has been reported in the United States. Respiratory isolation of suspected cases is well warranted, because pneumonic plague is in the differential diagnosis of HPS.

In general, the Sin Nombre and Black Creek Canal viruses were found to be sensitive *in vitro* to ribavirin. However, its efficacy in HPS cases is uncertain. Thus, a small number of patients with HPS has been treated with intravenous ribavirin in open-label protocol. Although the results of the trial showed 47% mortality rate in the ribavirin recipients compared with 50% in nontreated patients *(304)*, data should be interpreted with caution because most of the untreated patients were either ill during the early phase of the epidemic or presented in nonepidemic areas where the diagnosis may have been delayed.

Placement of a flow-directed pulmonary artery catheter for continuous measurement of cardiac output may be beneficial early in the clinical course. In addition, maintenance of low-normal pulmonary wedge pressures, compatible with satisfactory cardiac indices, is recommended because of the extreme capillary leak. Inotropic agents such as dobutamine, dopamine, and noradrenaline should be used earlier in the treatment of shock in HPS patients than in patients with

bacterial sepsis, together with judicious intravascular volume expansion with packed red blood cells to maintain delivery of oxygen if hemoglobin concentration falls *(304)*.

23.11.5.6 Baltimore Rat Virus

Disease caused by the Baltimore rat virus is characterized by acute onset of nausea, vomiting, fever, upper abdominal pain, protein in the urine, and hypertension. In some cases, liver and renal involvement occurs. Patients suffer fever, then 1 to 2 days of hypotension, followed by renal failure with liver enzyme derangements. Survivors can experience chronic renal impairment and disease.

23.12 Viral Hemorrhagic Fever: Arenaviruses

The Arenaviridae is a family of viruses whose members are generally associated with rodent-transmitted disease in humans. Each Arenavirus is usually associated with a particular rodent host species in which it is maintained. Arenavirus infections are relatively common in humans in some regions of the world and can cause severe illness (http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/arena.htm).

The family Arenaviridae consists of a single genus, Arenavirus, in which two serotypes are recognized: *LCM/Lassa complex (Old World arenaviruses)*, and *Tacaribe complex (New World arenaviruses)* (Table 23.3).

The first Arenavirus, the lymphocytic choriomeningitis virus (LCMV), was isolated in 1933 during a study of an epidemic of St. Louis encephalitis. By the 1960s, several similar viruses had been discovered and were all classified into a new family, Arenaviridae. Since Tacaribe virus was found in 1956, new arenaviruses have been identified every 1 to 3 years, on average. A number of these viruses are pathogenic to humans, causing hemorrhagic disease. The Junin virus, isolated in 1958, was the first of these to be recognized; it causes the Argentine hemorrhagic fever. In 1963, the Machupo virus, which causes the Bolivian hemorrhagic fever, was isolated, followed in 1969 by the Lassa virus in Africa. Most recently, the Guanarito and Sabia viruses, which cause the Venezuelan and Brazilian hemorrhagic fevers, respectively, were added to the family (http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/arena.htm).

The Junin, Machupo, Guanarito, and Sabia viruses, known to cause severe hemorrhagic fever, are included in the Category A Pathogen List as defined by the CDC and are listed as Biosafety Level 4 (BSL-4) agents *(279)*.

The hosts of arenaviruses are rodents: *Mastomys natalensis* for Lassa virus; *Mus musculus* for LCMV; *Calomys musculinus* for the Junin virus; *C. callosus* for the Machupo virus; *Zygodontomys brevicauda* and *Sigmodon alstoni* for the Guanarito virus, and *Neotoma* rodents for the Whitewater Arroyo virus. Transfer to humans occurs via contact with infected rodents or inhalation of infectious rodent excreta or secreta. The arenaviruses do not require arthropods for spread and do not infect insect cells. Hence, the human infection is *zoonotic*; that is, transmission is from animals to humans.

The Arenavirus particles are spherical to pleomorphic with a diameter ranging from 50 to 300 nm (average, 120 nm) *(354)*. They possess a dense lipid-containing envelope with 8- to 10-nm-long, club-shaped projections. When viewed in

Table 23.3 Arenaviruses (region of isolation is listed in parenthesis)

LCM/Lassa complex (Old World arenaviruses)	Tacaribe complex (New World arenaviruses)
Ippy virus (Central African Republic)	Amapari virus (Brazil)
Lassa virus (Lassa, Nigeria)[1]	Flexal virus (Brazil)
Lymphocytic choriomeningitis virus; LCMV (Missouri)[2]	Guanarito virus (Venezuela)[3]
Mobala virus (Central African Republic)	Junin virus (Argentina)[4]
Mopeia virus (Mozambique)	Machupo virus (Bolivia-Beni region)[5]
SPH 114202 virus	Parana virus (Paraguay)
	Pichinde virus (Colombia)
	Tacaribe virus (Trinidad)
	Sabia virus (Brazil)[6]
	Latino virus (Bolivia)
	Oliveros virus (Argentina)
	Pirital virus (Venezuela)
	Whitewater Arroyo virus (New Mexico)
	Tamiami virus (Florida)

[1]Pathogenic to humans: Lassa fever.
[2]Pathogenic to humans: lymphocytic choriomeningitis/aseptic (nonbacterial) meningitis.
[3]Pathogenic to humans: Venezuelan hemorrhagic fever.
[4]Pathogenic to humans: Argentine hemorrhagic fever.
[5]Pathogenic to humans: Bolivian hemorrhagic fever.
[6]Pathogenic to humans: Brazilian hemorrhagic fever.

cross section, the arenaviruses show grainy particles, which are ribosomes (20 to 25 nm) acquired from their host cells during the budding process (their function is still not yet understood). It is this characteristic that gave them their name (from the Latin *arenosus* meaning *sandy*).

The viruses are quickly inactivated at 56°C, at pH below 5.5 or above 8.5, or by exposure to UV and/or gamma irradiation.

23.12.1 Genome Structure of Arenaviruses

The arenaviruses possess a single-stranded RNA genome consisting of two segments: L (large, ~ 5.7 kb) and S (small, ~ 2.8 kb). Each segment forms a circle by hydrogen bonding of its ends. The naked genome is noninfectious. The negative-sense RNA is neither capped nor polyadenylated and is contained in a helical nucleocapsid that comprises two proteins: nucleocapsid (N) and RNA polymerase (L). The L segment encodes the L and Z proteins. The S segment's 5′ part is (+) sense and encodes the N protein; its 3′ part is (−) sense and encodes the G protein.

The envelope contains two glycoprotein spikes: GP1 and GP2. One unique property of the genomes of arenaviruses is their *ambisense* organization.

The most important viral antigens are the nucleoproteins and the glycoproteins, with the nucleoprotein antigens being the most conserved among the arenaviruses.

The replication of the arenaviruses involves two rounds of transcription—one before and one after the formation of the "reverse-sense" RNA intermediate, to cope with the ambisense coding strategy.

Ambisense Organization. Historically, the RNA viruses are classified based on the ability of their naked nucleic acid (RNA separated from the other viral components) to induce a lytic action. The procedure of transforming cells with viral nucleic acid is known as *transfection*. Eventually, it has been demonstrated that the ability to productively transfect a cell corresponded to the "sense" or polarity of the RNA. Viruses (such as the polio virus) whose genomes consist of message-sense (+, or positive sense) single-stranded RNA were the only ones found to be able to productively transfect permissive cells. The other viruses (such as influenza) were shown to have genomes that contained the complement to mRNA. In most cases, the missing factor was the absence of the viral polymerase that allowed the complementary RNA to be transcribed into message (http://virus.stanford.edu/arena/ambisense.htm).

Several complications with this classification system have arisen: (i) the retroviruses have been shown to contain a genome with single-stranded message-sense RNA that was not infectious. They require reverse transcriptase in order to carry out their complicated replication cycle. (ii) An analogous complication is the sense of the reoviruses, which contain message-sense RNA as part of their double-stranded genome. Enzymatic separation of the strands using a viral protein may be essential for translation. (iii) Some negative ssRNAs (the ssRNA is not infectious) were shown to display *ambisense*. Ambisense is a situation in which both the genome and its complement contain some coding information. Because translation *always* occurs in the 5′–3′ direction, the two strands are being translated in opposite directions, and because each strand has regions of + and − polarity, the viral organization/capability is defined as ambisense. Nevertheless, even though ambisense organization is seen in the arenaviruses (and also in the Bunyaviridae family's phleboviruses and tospoviruses), it is still convenient to classify the arenaviruses with the negative ssRNA viruses because they resemble them in both virion structure and infectivity (http://virus.stanford.edu/arena/ambisense.htm).

23.12.2 Human Significance of Arenaviruses

All South American arenaviruses were identified during the second half of the 20th century. However the arenaviruses seem to be quite ancient and have existed in nature for years as silent zoonotic foci *(354)*.

There have never been any reports of human-to-human transmission of LCMV. However, Machupo virus has clearly been responsible for severe nosocomial outbreaks, in which all cases were associated with a single index case that had returned from an endemic region *(354, 355)*. The common features of the reported nosocomial outbreaks include (i) the index case was critically ill and died; (ii) aerosol spread was the most likely explanation for the route of infection of at least some of the secondary cases; (iii) lethality was high; and (iv) transmission ceased after the secondary or tertiary cases *(354)*.

The case-fatality rate of the *Argentine hemorrhagic fever (AHF)* is approximately 20% in the absence of specific therapy. AHF is typically a seasonal disease with a peak of frequency occurring during the corn harvest (March to June). However, since the late 1980s, the epidemiology of AHF has been dramatically modified by the development of live-attenuated vaccine *(354)*.

The *Bolivian hemorrhagic fever (BHF)* was responsible for a large outbreak with a case-fatality rate of about 20% *(355)*. Most of the recorded infections were acquired by direct contact with rodents or by aerosol through infected excreta *(354)*.

The *Venezuelan hemorrhagic fever (VHF)* was recognized in 1989 and currently about 200 cases have been confirmed *(356)*. The number of reported human cases dropped between 1992 and 2002 despite the continuous circulation of the

virus in the rodent population during this period. However, a new outbreak was observed in 2002. The reason(s) for this hiatus and the secondary re-emergence of the disease are unknown *(354)*.

Very little is known about the health consequences of human infections with arenaviruses other than Junin, Machupo, Guanarito, Sabia, and LCMV *(354)*.

Pichinde virus has resulted in numerous seroconversions without any notable clinical significance *(357)*.

The Flexal and Tacaribe viruses have caused febrile illnesses in laboratory workers *(357–359)*. The Flexal virus has caused two symptomatic laboratory infections and should be considered as potentially dangerous *(358, 359)*.

Tacaribe virus has resulted in a single case of febrile illness with mild central nervous system symptoms *(358)*.

In 1999 and 2000, three fatal cases of illness were reported in California, and their association with the Whitewater Arroyo virus was based on PCR and sequencing results *(360)*. The patients infected with the Whitewater Arroyo virus were healthy prior to the viral infection and there was no history of travel outside California during the 4 weeks preceding the illness. In one of the cases, the virus was probably acquired via the aerosol pathway during the removal of rodent droppings from the home.

23.12.3 Transferrin Receptor 1

At least five arenaviruses cause viral hemorrhagic fever in humans. One of these pathogens, the Lassa virus (an Old World Arenavirus), together with the lymphocytic choriomeningitis virus (LCMV) use the cellular receptor α-dystroglycan as the host cellular receptor to infect cells *(361)*. The South American hemorrhagic fever viruses (New World arenaviruses), the Machupo, Guanarito, Junin, and Sabia viruses, do not use the α-dystroglycan receptor. Instead, a specific, high-affinity association between the transferring receptor 1 (TfR1) and the entry glycoprotein (GP) of the Machupo virus has recently been established, demonstrating that TfR1 is the host cellular receptor of the four South American arenaviruses *(362)*. In addition, it has also been shown that expression of TfR1 in hamster cell lines efficiently enhanced the entry of retroviruses expressing the GP of the Machupo virus. Expression of transferring receptor 2 (TfR2) in the same cell lines did not promote Arenavirus infection. This confirmed that TfR1 is a necessary cellular receptor for all New World arenaviruses, and anti-TfR1 antibody was shown to efficiently inhibit infection in all cases *(362)*.

As TfR1 is normally involved in the cellular iron transport, further experiments have been directed at studying the effect of iron on the Arenavirus infection process. When the cell culture medium was depleted of iron, which is known to upregulate the expression of TfR1, there has been a significant enhancement in the efficiency of infection by both the Junin and Machupo viruses. Conversely, supplementation of the medium with iron (and the subsequent downregulation of TfR1 expression) inhibited the Junin and Machupo virus infection *(362)*.

Several properties of TfR1 indicated its possible role in arenaviral replication and disease. Thus, TfR1 was rapidly and constitutively endocytosed to an acidic compartment, which is consistent with the pH dependence of Arenavirus entry *(363)*. Further, it is expressed ubiquitously and at high levels on activated or rapidly dividing cells, including macrophages and activated lymphocytes, major targets of arenaviral infection *(364, 365)*. TfR1 has also been highly expressed on endothelial cells *(366, 367)*, which are thought to be central to the pathogenesis of hemorrhagic fever *(368)*.

TfR1 is a homodimeric type II transmembrane protein comprising a small cytoplasmic domain, a single-pass transmembrane region, and a large extracellular domain. The crystal structure of the butterfly-shaped dimeric TfR1 ectodomain showed that each monomer has three structurally distinct domains: a protease-like domain proximal to the membrane, a helical domain accounting for all the dimer contacts, and a membrane-distal apical domain *(369)*.

Although much is understood of the transferring endocytotic cycle, little has been uncovered of the molecular details underlying the formation of the TfR1 receptor-transferrin complex. Using cryoelectron microscopy, a density map of the TfR1-transferrin complex at subnanometer resolution has recently been produced *(370)*.

The role of iron in this process, the possible consequences of iron deficiency, and the therapeutic potential of a humanized anti-TfR1 antibody remain important directions for future research *(362)*. To this end, several anti-human TfR1 antibodies have already been developed and are currently being investigated as antitumor therapeutics *(365)*. Some of these antibodies, including the anti-TfR1 antibody, did not compete with transferrin, indicating that an anti-TfR1 antibody can limit arenaviral replication in an infected individual without interfering with iron metabolism *(362)*.

23.12.4 Arenaviruses Hemorrhagic Fever: Clinical Manifestations

The clinical manifestations of the South American hemorrhagic fevers are nearly identical regardless of the virus responsible for the disease *(356, 358, 371)*.

The incubation period is typically from 7 to 14 days, with extreme cases extending from 5 to 21 days. Secondary infection to very high-load inoculum may result in the reduction of the incubation period to 2 days.

The onset is gradual, with fever and malaise, followed by myalgia, back pain, headache, and dizziness. Hyperesthesia of the skin is common. However, the most important clinical features of the disease are hemorrhagic and neurologic. They can present separately or combined *(354)*.

The hemorrhagic manifestations, which include petechiae of the skin and hemorrhaging from the gums, vagina, and the gastrointestinal tract, and will typically start around the fourth day of illness, indicate the advent of hypovolemic clinical shock. The blood loss is usually minor, so the hematocrit generally will increase as the capillary leak syndrome, the hallmark of the disease, becomes more severe. Bleeding and prothrombin time may be prolonged, and reduction of factors II and VII of the coagulation cascade have been observed. The renal function is generally delayed until shock occurs, but the urinary protein may be high. Pronounced thrombocytopenia may also occur *(354)*.

Neurologic manifestations, presenting as tremor of the hands and an inability to swallow or to speak clearly, may develop, and these can progress to grand mal convulsions, coma, and death in the absence of significant capillary leak or hemorrhagic signs. Death usually occurs between 7 and 12 days after the onset.

Symptoms that appear to be more specifically associated with one or another of the viruses have been reported *(372)*. For example, while frequency of clinical and laboratory findings were identical for Junin and Machupo virus infections, there have been clear differences with the Guanarito infections: pharyngitis, vomiting, and diarrhea were more frequently observed with the Guanarito virus; in contrast, petechiae, erythema, facial edema, hyperesthesia of the skin, and shock were frequently observed in the case of Junin or Machupo infections. A fatal outcome of Junin virus infection has been more frequently observed in pregnant women in the last trimester, and a high fetal mortality rate was associated with both Junin and Machupo virus infections *(373)*.

In the three fatal cases associated with the Whitewater Arroyo virus, the onset was characterized by nonspecific febrile symptoms including fever, headache, and myalgia *(360)*. All patients presented with acute respiratory distress syndrome, and two developed liver failure and hemorrhagic manifestations. Death occurred within 8 weeks after the onset. The direct implication of the Whitewater Arroyo virus in the pathophysiology of these clinical cases remains to be formally established *(354)*.

23.12.4.1 Diagnosis and Treatment

Arenaviruses can be isolated by propagating in cell cultures (particularly in Vero cells). However, because the cytopathic effect is inconsistent, virus-infected cells are usually detected by direct immunofluorescent tests *(354)*.

Arenaviruses can be isolated from serum and throat washings collected 3 to 10 days after onset; less frequently from urine. Specifically, the Machupo virus is recovered from only 20% of acute-phase sera and even less frequently from throat washings or urine *(354)*. With regard to the Junin virus, reverse transcriptase–polymerase chain reaction (RT-PCR) is to date the only method available for rapid diagnosis.

It should be emphasized that arenaviruses are not recovered from the cerebrospinal fluid (CSF) of patients presenting with CNS symptoms when infected with the South American viruses. Viral RNA can be detected by RT-PCR from serum, plasma, urine, throat washings, and various tissues, and the sequencing of the amplified region can be used to identify the implicated virus. The RT-PCR diagnosis offers the advantage of reducing the delay to response and demonstrates a greater sensitivity compared with that of cell culture. Additional techniques for direct detection of viral presence in tissues include *in situ* nucleic acid hybridization and antigen detection ELISA *(354)*.

Indirect serologic diagnosis is based on the detection of antibodies to the nucleoprotein and/or the envelope glycoproteins. Immunofluorescence (IF) tests and ELISA methods using lysates of infected cells would permit the detection of antibodies to the nucleoprotein. However, because this antigen is the most conserved among the arenaviruses, cross-reactions are frequent. Nevertheless, together with ELISA, the indirect IF tests remain simple, inexpensive, rapid, and sensitive assays for detecting Arenavirus infection. The precise identification of the viral species involved should be based on neutralization tests *(354)*.

For the South American viruses causing hemorrhagic fever, the differential diagnosis principally includes yellow fever, dengue hemorrhagic fever, viral hepatitis, leptospirosis, hemorrhagic fever with renal syndrome caused by hantaviruses, rickettsial diseases, sepsis with disseminated intravascular coagulation, and, in the case of CNS involvement, viral encephalitis *(354)*.

Nonspecific treatment of Arenavirus hemorrhagic fever has been based on monitoring and correcting fluid, electrolyte, and osmotic balance. Hemorrhaging can be treated with clotting factor and/or platelet replacement; however, the most serious symptoms usually are caused by capillary leakage *(354)*.

Immune Therapy

The case-fatality ratio of the Argentine hemorrhagic fever, which is about 20% without specific treatment, has been decreased to less than 1% when patients were treated with convalescent serum *(371)*, especially when the immune serum therapy was given within the first 8 days of illness

(374, 375). There is experimental evidence suggesting that the immune plasma may work through viral neutralization.

In the case of Machupo viral hemorrhagic fever, in spite of the recognized efficacy of the convalescent plasma, the low number of cases since its initial outbreak and the absence of a program for collecting and storing of Bolivian immune plasma creates the possibility of future shortages of plasma *(354)*. Hence, antiviral-based approaches may hold a better promise for Bolivian hemorrhagic fever (BHF) treatment.

Ribavirin

The primary target of ribavirin is thought to be the IMP dehydrogenase, an enzyme that converts inosine 5′-monophosphate (IMP) into xanthosine-5′-monophosphate (XMP) *(376)*. The viral RNA synthesis is reduced by ribavirin through a marked decrease in the levels of guanosine-5′-monophosphate (GMP), guanosine diphosphate (GDP), and guanosine-5′-triphosphate (GTP). The reduction of the GTP pool level is the result of a decrease in the 3′,5′-monophosphate (ATP) pool level, because GTP acts as a cofactor for the conversion of IMP into succinyl AMP by the adenylsuccinate synthethase. Typically, the ribavirin treatment will lead to the rise of the intracellular pool level by (i) directly inhibiting the IMP dehydrogenase; (ii) indirectly inhibiting the adenosuccinyl synthetase subsequent to the reduced GTP pool levels; (iii) directly interfering with viral RNA synthesis *(377)*; (iv) directly interfering with the viral mRNA capping the guanylation process; and *(v)* by directly interfering with the generation and elongation of the primer during the viral RNA transcription *(354)*.

The main side effects of ribavirin include thrombocytosis and severe anemia, which resolve after the administration has been stopped.

In 1994, two patients infected with the Machupo virus were treated with ribavirin *(378)*; both patients recovered without sequelae. In 1995, a case of a laboratory-acquired Sabia virus infection occurred in the United States. The patient was treated intravenously with ribavirin 3 days after the infection and recovered without any sequelae *(379)*. These and other (mainly *in vitro*) studies against arenaviruses have shown them to be susceptible to ribavirin *(354)*.

The protocols for ribavirin administration were defined for Lassa virus infections. With regard to its therapeutic use, ribavirin is usually given intravenously at a loading dose of 30 mg/kg, then 16 mg/kg every 6 hours for 4 days, and then 8 mg/kg every 8 hours for 6 days *(279)*. Concerning its prophylactic use for at-risk populations, the recommended dose regimen is oral administration of 500 mg every 6 hours for 7 days *(279)*. Although there is no specific information for South American hemorrhagic fever arenaviruses, the ribavirin dose regimens recommended for Lassa fever may still apply *(354)*.

Hemorrhagic Fever Vaccines

The Argentine hemorrhagic fever is the only South American Arenavirus hemorrhagic fever for which extensive studies have been conducted to develop and evaluate a candidate vaccine *(354)*. Early attempts were based on the Tacaribe virus to elicit protection against a lethal challenge with the Junin virus *(380, 381)*.

Recently, a new vaccine, known as Candid 1, has been developed based on live-attenuated Junin virus *(382)*. Studies in non-human primates (*Macacus rhesus*) have shown full protection against a virulent strain of the Junin virus, most likely by inducing a neutralizing antibody response *(383)* coupled with the development of a virus-specific antibody-dependent cellular cytotoxicity *(384)*. The immunogenicity and safety of Candid 1 have been tested in rhesus macaques by subcutaneous administration at increasing doses. The results have shown no clinical and/or biological adverse reaction, and the Candid 1 is being considered for evaluation in Phase I/II human trials *(385)*.

The neutralizing antibody titers to Candid 1 vaccine against Argentine hemorrhagic fever (AHF) were studied for 2 years after vaccination in volunteers to assess whether the kinetics and/or magnitude of this immune response had been modified by previous infection with the Junin virus and lymphocytic choriomeningitis (LCM). The overall results of this study indicated that the immune response to Candid 1 boosted preexisting immunity to Junin virus but was not changed by previous experience with the LCM virus *(386)*.

Whether Candid 1 vaccine may prove useful to cross-protect populations against the Bolivian hemorrhagic fever infection—and possibly against the Venezuelan hemorrhagic fever—needs to be further explored. Cross-reactivity of Junin virus with other arenaviruses was found to be restricted to nucleoprotein-specific monoclonal antibodies and occurred only with New World arenaviruses *(387)*. Cross-reactivity also showed that the Junin virus was most closely related to the Machupo and Tacaribe viruses.

23.12.5 Lassa Fever

Lassa fever has been found predominately in West Africa, especially in Nigeria, Sierra Leone, and Liberia but was also reported in other West African countries. The actual number of cases must run into the thousands, and the impact of the disease must be considerable. Thus, a study carried out in Sierra Leone from 1977 until 1979 reported that 12% of all

hospital admissions to just two hospitals were Lassa fever. Furthermore, deaths due to Lassa fever represented 30% of all hospital deaths. Seroprevalence to Lassa fever was found in 7% of all febrile episodes studied. The multimammate rat (*Mastomys natalensis*) appears to be the natural reservoir, and these rodents sustain chronic viremia and virus shedding in the absence of histologic lesions. Human-to-human transmission has been extensively documented. Lassa fever is also a well-recognized nosocomial infection (http://virology-online.com/viruses/haemorrhagicfever.htm).

The Lassa virus is typically spread through aerosolized particles, via either infected rodents or close contact with infected individuals. Additional contact with infected bodily fluids, including blood, urine, and vomit, has been known to spread the virus.

23.12.5.1 The α-Dystroglycan Receptor

α-Dystroglycan (α-DG), a peripheral membrane protein, has been identified as the receptor of both the Lassa fever virus and the lymphocytic choriomeningitis virus (LCMV) *(361)*.

The α-dystroglycan, which was found to be interactive with LCMV, has been purified from cells permissive to infection. Tryptic peptides from this protein were determined to be α-dystroglycan. Several strains of LCMV and Lassa fever virus (LFV) bound to α-dystroglycan, and soluble α-DG blocked both LCMV and LFV infection. Cells bearing a null mutation of the gene encoding α-DG were resistant to LCMV and LFV infections, and reconstitution of α-DG expression in null mutant cells restored the susceptibility to LCMV and LFV infections, thus demonstrating that α-DG is the cellular receptor for both viruses *(388)*.

23.12.5.2 Lassa Fever: Diagnosis and Clinical Manifestations

The incubation period of Lassa virus is between 5 and 21 days, with symptoms usually appearing 10 days after infection.

Traditional laboratory tests provide little help in the way of diagnosis. Leukocyte levels and platelet counts are not useful means of diagnosis. Albuminuria is common. The aspartate aminotransferase (AST) levels parallel the amount of virus in the blood, which is a useful factor in determining the prognosis: the greater the amount of virus in the blood, the more likely the associated disease will be fatal. The Lassa virus is easily isolated from the blood during the febrile stage of the illness, and complement fixation (CF), immunofluorescent antibody assay (IFA), and ELISA may all be useful for detecting viral antibodies (http:// stanford.edu/group/virus/arena/2005/lassavirus.htm). The antigen-capture ELISA test detects virus antigen or virus-specific antibodies (either IgM or IgG). Using a combination of antigen and IgM antibody tests will allow virtually all Lassa virus infections to be diagnosed early.

Lassa fever typically begins with sore throat, fever, chills, headache, myalgia, and malaise, which may be followed by anorexia, vomiting, and chest pains. Severe abdominal pain and swelling of the neck and face are common. Hemorrhages develop in about 15% to 20% of patients, predominately in the mucous membranes, and are associated with significant rise in mortality. Other symptoms may also include maculopapular rash, cough, diarrhea, conjunctivitis, tremors, dizziness, or signs of hepatitis.

Neurologic phenomena are less common but are nevertheless important. Aseptic meningitis, encephalitis, and global encephalopathy with seizures have all been documented in cases of Lassa virus infection.

Although unusual, deafness is a common feature during late-stage illness or early convalescence and may be either ephemeral or permanent.

When treated in a hospital setting, the mortality rate of Lassa fever is between 15% and 20%. However, it increases dramatically (up to 60%) in areas where appropriate medical care is unavailable. Fatal cases of Lassa fever rarely show any signs of remission, progressing from fever to shock to death in an unrelenting slide. Survivors remain symptomatic for approximately 2 to 3 weeks after the onset of symptoms.

Lassa fever infections in pregnant women are generally more serious—mortality rates range from 30% to 50%, and 95% of the pregnancies end in abortion.

23.12.5.3 Treatment of Lassa Fever

Treatment of Lassa fever is largely symptomatic. Good supportive care is required in the case of severe illness, and shock must be managed very carefully. Management of bleeding and hydration is critical.

Ribavirin has been shown to be effective against Lassa fever, with a two- to threefold decrease in mortality in high-risk patients. Treatment with intravenous ribavirin should commence as soon as possible. Protocols for ribavirin administration were defined for Lassa virus infections. In regard to its therapeutic use, ribavirin is usually given intravenously at a loading dose of 30 mg/kg, then 16 mg/kg every 6 hours for 4 days, and then at 8 mg/kg every 8 hours for 6 days *(279)*. Concerning its prophylactic use for at-risk populations, the recommended dose regimen is oral administration of 500 mg every 6 hours for 7 days *(279)*. However, the drug was shown to be embryotoxic in animal models, and its use in pregnant women is contraindicated.

Postexposure prophylaxis against Lassa fever may be desirable. However, there is no established safe prophylaxis against the disease. Various combinations of hyperimmune

convalescent immunoglobulin and/or oral ribavirin may be considered.

At present, there is no effective vaccine available. The development of inactivated vaccines has been hampered by the inability to obtain large quantities of virus from cell cultures. This problem has redirected research interest toward live-attenuated vaccines.

23.12.6 Lymphocytic Choriomeningitis Virus

The LCMV is the prototype member of the Arenaviridae. The LCMV rarely infects humans and when it does, it is usually under conditions when the indigenously infected mouse populations are extremely dense or from contact with infected animals. Individuals become infected with LCMV after exposure to fresh urine, droppings, saliva, or nesting materials. Transmission may also occur when these materials are directly introduced into broken skin, the nose, eyes, or the mouth, or presumably via the bite of an infected rodent. Person-to-person transmission has not been reported with the exception of vertical transmission from an infected mother to the fetus (http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/lcmv/qa.htm). Recent investigations have indicated that organ transplantation may also be a means of transmission *(389)*.

In spite of an apparent decrease in occurrence, the LCMV continues to circulate in rodents and to infect humans as established by seroepidemiologic studies *(390–392)*.

Based on available data for LCMV infections, immunofluorescence (IF) assays appear to be best suited for making a rapid diagnosis soon after infection *(354)*. Neutralizing antibodies appear relatively late after infection and therefore cannot be recommended for detection of seroconversion early in convalescence. However, because the neutralizing antibodies persist for many years (presumably lifelong), they seem to be well suited for confirmation of unexpected results and for detection of infection from distant past. The complement fixation (CF) tests have been considered of little value for the serologic diagnosis of LCMV *(392a)*.

LCM disease is typically mild and is usually manifested as a form of aseptic meningitis (lymphocytic meningitis) or an influenza-like illness. During the first phase of the disease, the most common laboratory abnormalities are a low white blood cell count (leukopenia) and a low platelet count (thrombocytopenia). After the onset of the neurologic disease during the second phase, an increase of the protein levels, an increase in the number of white blood cells, or a decrease in the glucose levels in the CSF have been observed. CNS complications beyond aseptic meningitis include encephalitis and may involve cranial nerve palsies and/or damage to the autonomic nervous system. Non-CNS complications include orchitis, myocarditis, and small-joint arthritis—these symptoms developed, if at all, in the late stage of the disease during the recurrence of fever.

Intrauterine infection with LCMV may affect the fetus leading to hydrocephalus and chorioretinitis with persistent spastic pareses and death within several years.

On very rare occasions, severe or fatal LCM disease is seen with hemorrhagic manifestations.

The LCMV may also be pathogenic to the fetus leading to abortion or resulting in delivery of babies with hydrocephalus (increased fluid in the brain), chorioretinitis, and mental retardation.

A peripheral membrane protein, α-dystroglycan (α-DG), has been identified as the cellular receptor for both the lymphocytic choriomeningitis and Lassa fever viruses (see Section 23.12.5.1) *(361)*.

LCM disease requires no more than symptomatic treatment.

23.13 Viral Hemorrhagic Fever: Flaviviruses

The Flaviviridae is a family of more than 66 viruses, with nearly 30 of these viruses associated with human disease. Flaviviruses associated primarily with hemorrhagic fever include the yellow fever virus, dengue hemorrhagic fever virus, Kyasanur Forest virus, and the Omsk hemorrhagic fever virus.

The flaviruses represent single-strand, (+)-sense RNA, enveloped, spherical viruses, 40 to 60 nm in diameter. Their genome has a 5′ cap but is not polyadenylated at the 3′ end. The entire Flavivirus genome is translated as a single polyprotein, which is then cleaved into the mature proteins. Complementary (−)-strand RNA is synthesized by NS proteins and used as a template for genomic progeny RNA synthesis. The first mechanism for (−)-strand RNA synthesis described in Flavivirus has been proposed, in which the promoter element functions from the 5′ end of a circular viral genome. Because similar 5′ − 3′ long-range interactions were observed in many viral RNA genomes, the proposed mechanism for dengue virus may represent a wide-spread strategy for viral RNA replication *(393)*.

Although the mechanisms of RNA replication of (+)-strand RNA viruses is still unclear, in a recent study, the first promoter element for Flavivirus RNA synthesis has been identified using dengue virus as a model *(393)*.

Flavivirus assembly occurs during budding, characteristically into cytoplasmic vacuoles rather than at the cell surface. Release occurs when the cell lyses.

Many Flavivirus species can replicate in both mammalian and insect cells and survive for long periods in their hosts, such as ticks, by replicating in the insect without causing damage.

23.13.1 Yellow Fever

Yellow fever is a viral disease of short duration and varying degree of severity that is transmitted primarily by mosquitoes. The infection caused by the yellow fever Flavivirus is so named because of the yellow skin color (jaundice) observed in persons with serious illness. The disease has been associated with tropical climates (reported in 33 countries worldwide) and caused large epidemics in Africa (90% of all cases) and the Americas (10%). To date, yellow fever has not been reported in Asia and Australia, where very strict quarantine regulations exist as well as a requirement of a valid vaccination certificate for people traveling from areas in Africa and South America where yellow fever occurs (the disease is covered by the International Quarantine Regulations). Although an effective vaccine has been available for 60 years, the number of people infected over the past two decades has increased, and yellow fever is again recognized as a serious public health threat (http://www.who.int/mediacentre/factsheets/fs100/en).

In Africa, there are two distinct genetic types (known as topotypes) associated with East and West Africa. In South America, two different types have been defined, but since 1974 only one has been identified as the cause of disease outbreaks.

Transmission. Humans and monkeys are the principal species to be infected. The virus is transmitted by a biting mosquito (horizontal transmission). The mosquito can also pass the virus via infected eggs to its offspring (vertical transmission). The eggs are resistant to drying and lie dormant through dry conditions, hatching when the rainy season begins, thus making the mosquito the true reservoir of the virus, ensuring transmission from one year to the next.

Female mosquitoes transmit the virus because they take blood from the bitten victim. Male mosquitoes do not take blood from the victim. After the mosquito ingests the virus, it takes about a week to 10 days or so for that mosquito to become infective.

Several different species of the *Aedes* (*Ae. africanus* in Africa) and *Haemagogus* (in South America only) mosquitoes transmit the yellow fever virus. These mosquitoes are either domestic (i.e., they breed around houses), wild (they breed in the jungle), or semidomestic (they display mixture of habits). Any region populated with these mosquitoes can potentially harbor the disease.

23.13.1.1 Yellow Fever: Infections to Humans

There are three types of transmission cycles for the yellow fever: sylvatic, intermediate, and urban. All three cycles exist in Africa, but in South America, only the sylvatic and urban cycles occur (http://www.who.int/mediacentre/factsheets/fs100/en):

(i) *Sylvatic (or Jungle) Yellow Fever*. In the tropical rainforest, yellow fever occurs in monkeys that are infected by wild mosquitoes. The infected animals can pass the virus to other mosquitoes that feed on them. The infected wild mosquitoes can then bite humans, causing sporadic cases of yellow fever. On occasion, the virus spreads beyond the infected individual.

(ii) *Intermediate Yellow Fever*. In the humid or semihumid savannahs of Africa, small-scale epidemics occur. These epidemics behave differently than do the urban epidemics, in that many separate villages in an area suffer cases simultaneously, but fewer people die from infection. The semidomestic mosquitoes infect both monkeys and human hosts. Such areas are often called the *zone of emergence*, where increased contact between humans and infected mosquitoes leads to disease. The intermediate yellow fever is the most common type of outbreak seen in recent decades in Africa. It can shift to a more severe urban-type epidemic if the infection is carried into a suitable environment populated with domestic mosquitoes and unvaccinated humans.

(iii) *Urban Yellow Fever*. This type of disease, causing large epidemics of yellow fever, occurs only in humans. Large epidemics can take place when migrants introduce the virus into areas with high human density. Domestic mosquitoes (*Aedes aegyptii*) feed on humans and carry the virus from person to person, with no monkeys involved in the transmission. These outbreaks tend to spread outwards from one source to cover a wide area.

The disease is diagnosed by a blood serum or tissue tests, antigen detection by immunofluorescence or immunochemistry, RNA detection by RT-PCR, or by specific antibody detection.

23.13.1.2 Yellow Fever: Clinical Manifestations

After an incubation period of 3 to 6 days, 5% to 50% of infected people will develop the disease. The signs and symptoms of yellow fever can be divided into three stages. The early stage is characterized by headache, muscle pain, fever, loss of appetite, vomiting, and jaundice. Transient viremia and primary multiplication in lymph nodes takes place. The second stage is a period of remission where fever and other symptoms resolve—most individuals recover at this stage. However, up to 50% of infected individuals may move onto the third, most dangerous, stage, which is characterized by a multiorgan dysfunction, especially liver and kidney failure, epistaxis, other bleeding disorders such

as hemorrhage, disseminated intravascular coagulation, and brain dysfunction including delirium, seizures, coma, and shock; up to 30% of patients will die. Overall, the mortality rate of yellow fever is 40% in severe cases. Genetic variations between different human populations result in different severity of disease; however, the genes involved are still not known (http://microbiologybytes.com/virology/flsaviviruses.html).

23.13.1.3 Yellow Fever: Treatment

At present, there are no antiviral drugs effective against yellow fever. Serious cases of yellow fever *always* need hospital treatment, which is symptomatic—mainly intravenous rehydration with fluids and salts to restore the electrolyte balance. Paracetamol should be avoided if there is already evidence of liver damage. In mild cases, the pain may be relieved with painkiller (analgesic) drugs and high temperature with antipyretics.

23.13.1.4 Yellow Fever Vaccines

Vaccination against yellow fever is the best way of avoiding the disease and doing so is *absolutely recommended*. The vaccine is almost 100% effective against yellow fever, and its tolerance is excellent. It provides protection against the disease for at least 10 years beginning 10 days after its administration.

The yellow fever *17D vaccines* are based on a live-attenuated strain, first developed in 1937 from a parent Asibi strain *(394)* and manufactured with the approval of WHO in 11 countries after standards for its production had been approved, including the adoption of a seed lot system *(395)*. Mass vaccination was conducted in the late 1930s and early 1940s in Brazil, various countries in Africa, and the armed forces of the United States and its allies.

Vaccines currently in use are derived from two distinct substrains (17DD at the 284th to 286th passage level; and 17D-204 at the 233rd to 237th passage level), which represent independently maintained series of passages from the original 17D virus developed in 1937 *(396)*.

The 17D vaccine is administered at 0.5 mL subcutaneously and requires a booster every 10 years.

Adverse reactions include hypersensitivity to eggs, encephalitis in the very young, hepatic failure, as well as rare reports of deaths from massive organ failure. In June 2001, seven cases of yellow fever vaccine–associated viscerotropic disease (YEL-AVD; previously known as multiple organ system failure) were reported as the result of vaccination with 17D-derived yellow fever vaccine *(338–340,397,400)*. These and other reports on two suspected cases of YEL-AVD, and four cases of suspected YEL-associated neurotropic disease (YEL-AND; previously called postvaccinal encephalitis), prompted the Advisory Committee on Immunization Practices (ACIP) at the CDC to recommend enhanced surveillance for adverse effects after vaccination with the 17D vaccine *(401)*.

However, the risk to unimmunized individuals either living in or traveling to areas where there is known yellow fever transmission is far greater than the risk of having a vaccine-related adverse event, and WHO policy on yellow fever vaccination remains unchanged, strongly recommending vaccination against yellow fever.

Contraindications. Administration of yellow fever vaccines has been contraindicated for certain populations, as follows (http://www.cdc.gov/ncidod/dvbid/yelloefever.html):

(i) The vaccine should never be given to infants under 6 months of age because of a risk of viral encephalitis. Most vaccinations should be deferred until the child is 9 to 12 months of age.
(ii) Pregnant women should not be vaccinated because of the potential risk that the developing fetus may become infected from the vaccine.
(iii) Individuals hypersensitive to eggs should not receive the vaccine because it is prepared in embryonated eggs. In case of questionable history of egg hypersensitivity, an intradermal test dose may be administered under medical supervision.
(iv) Individuals with immunosuppression associated with HIV infection or malignancies or those who are receiving immunosuppressive therapy and/or radiation should not receive the vaccine. Patients with asymptomatic HIV infection may be vaccinated if exposure to yellow fever cannot be avoided.

23.13.2 Dengue Fever

Dengue fever was first described in 1779–1780, and the virus was isolated in 1944 *(402)*. The disease pattern associated with dengue-like illness from 1780 to 1940 was characterized by relatively infrequent but often large epidemics. However, during that time, the dengue viruses seemed to become endemic in many tropical urban centers *(403)*.

The ecologic disruption in the Southeast Asia and the Pacific regions during and after World War II created optimal conditions for increased transmission of mosquito-borne disease and the beginning of a global pandemic of dengue, followed in 1953–1954 by an epidemic of the newly identified dengue hemorrhagic fever (DHF) in the Philippines *(404, 405)*. In Asia, epidemic dengue hemorrhagic fever expanded geographically from Southeast Asian countries west to India, Sri Lanka, the Maldives, and Pakistan, and

east to China *(406)* and the Pacific *(407)*. The dengue hemorrhagic fever has emerged as the leading cause of hospitalization and death among children in many Southeast Asian countries *(403)*.

After the eradication of the dengue virus mosquito-vector *Aedes aegyptii*, epidemics of the disease abated. However, after the discontinuation of the eradication program in the early 1970s, this species started to reinvade the countries from which it had been eradicated, and epidemics of dengue invariably followed the re-infestation of a country by *Ae. aegyptii (405, 408)*.

Before the 1980s, there was little information about the distribution of dengue viruses in Africa. Since then, however, major epidemics caused by all four serotypes have occurred in both East and West Africa *(407)*.

Each year, cases of dengue disease are imported to the continental United States and documented by the CDC. These cases represent the introductions of all four serotypes from all tropical regions of the world reflecting the increased number of people traveling from and to those areas, thereby making the potential for epidemic dengue transmission in the United States a possibility. After an absence of more than 40 years, autochthonous transmission, secondary to importation of the virus in humans, occurred on several occasions during the 1980–1997 period *(403, 409)*. Although small, these outbreaks still underscore the potential for dengue transmission in the United States where two competent mosquito vectors (*Ae. aegyptii* and *Ae. albopictus*) exist and can transmit dengue to humans, thereby increasing the risk of autochthonous dengue transmission, secondary to imported cases *(405, 410)*.

The dengue fever virus belongs to the genus Flavivirus of the family Flaviviridae, which contains approximately 70 species *(411)*. It comprises four serotypes, known as DEN-1, DEN-2, DEN-3, and DEN-4. Infection with one dengue serotype provides lifelong immunity to that virus, but since there is no cross-protective immunity to the other serotypes, persons living in an area of endemic dengue can be infected with all four dengue serotypes during their lifetimes *(405)*.

Transmission Cycles. The primitive enzootic transmission cycle of the dengue viruses involves canopy-dwelling *Aedes* mosquitoes and lower primates in the rainforests of Asia and Africa *(405)*. Current evidence suggests that these viruses do not regularly move out of the forests to urban areas *(412)*. The most important transmission cycle from a public health standpoint is the urban endemic/epidemic cycle in large urban centers of the tropical regions. The viruses are maintained in an *Ae. aegyptii*-to-human-to-*Ae. aegyptii* cycle with periodic epidemics. Often, multiple virus serotypes cocirculate in the same urban area (hyperendemicity) *(403)*.

Humans are infected with dengue viruses by the bite of an infected female mosquito. The principal vector, *Aedes aegyptii*, is a small, black-and-white, highly domesticated tropical insect that prefers to lay its eggs in artificial containers in immediate proximity to homes. The adult mosquitoes usually rest indoors, are unobtrusive, and prefer to feed on humans during the daylight hours; they infect several persons in one single blood meal. This feeding pattern makes *Ae. aegyptii* an efficient epidemic vector *(403)*.

23.13.2.1 Dengue Fever Virus Infections: Diagnosis and Clinical Manifestations

Primary infection may be asymptomatic or may result in dengue fever. The incubation period after a bite by an infected mosquito is 3 to 14 days (average, 4 to 7 days) *(403)*.

During the acute initial febrile period, which may be as short as 2 days and as long as 10 days, dengue viruses may circulate in the peripheral blood. If other *Ae. aegyptii* mosquitoes bite the already ill person during this febrile viremic stage, those mosquitoes may become infected and subsequently transmit the virus to other uninfected persons, after an extrinsic period of 8 to 12 days *(405, 413)*.

Dengue virus infection in humans causes a spectrum of illnesses ranging from asymptomatic or mild febrile illness to severe and fatal hemorrhagic disease *(403)*. Infection with any of the four dengue serotypes will elicit a similar clinical presentation, which may vary in severity depending on a host of risk factors, such as the strain and serotype of the infecting virus and the person's immune status, age, and genetic background *(405, 414–416)*.

Dengue Fever

The classic dengue fever is primarily a disease of older children and adults and may vary with age. Infants and young children develop rash and flu-like symptoms, whereas older children and adults may present with high fever and more severe symptoms.

After the end of the incubation period, the person may experience acute onset of fever accompanied by a variety of nonspecific signs and symptoms, such as headache and body aches, retro-orbital pain, nausea and vomiting, arthralgia (pain in the joints) that can progress to arthritis (inflammation of the joints), myositis (inflammation of muscle tissue), and a discrete macular or maculopapular rash. Patients may be anorexic, have an altered taste sensation, and have mild sore throat *(405, 417, 418)*. In this situation, clinical differentiation from other viral diseases may not be possible.

The initial temperature may rise to 102°F to 105°F, and fever may last for 2 to 7 days. The fever may drop after a few days, only to rebound 12 to 24 hours later (saddleback). Lymphadenopathy is common. A second rash may appear between days 2 and 6 of illness.

Hemorrhagic manifestations in dengue fever are not uncommon and range from mild to severe. Hematuria occurs infrequently, and jaundice is rare *(403)*.

Dengue fever is generally self-limiting and is rarely fatal. Whereas the acute phase lasts between 3 and 7 days, the convalescence phase may be prolonged for weeks and may be associated with weakness and depression, especially in adults.

Dengue Hemorrhagic Fever

Dengue hemorrhagic fever (DHF) is primarily a disease in children under 15 years of age, although it may also occur in adults *(419)*. It is characterized by sudden onset of fever, which usually lasts for 2 to 7 days, and a variety of non-specific signs and symptoms. During the acute phase of the illness, it is difficult to differentiate between DHF and dengue fever, as well as other diseases found in tropical settings. However, as fever remits, characteristic manifestations of plasma leakage appear, making accurate clinical diagnosis possible in many cases *(403)*.

The critical stage of DHF is at the time of defervescence, but signs of circulatory failure or hemorrhagic manifestations may occur from about 24 hours before to 24 hours after the temperature falls to normal or below. There is evidence of vascular leak syndrome, and common hemorrhagic manifestations include skin hemorrhages (petechiae, purpuric lesions, and ecchymoses). Epistaxis, bleeding gums, gastrointestinal hemorrhages, and hematuria occur less frequently.

Dengue Shock Syndrome

Dengue shock syndrome (DSS) is usually a progression of dengue hemorrhagic fever and is often fatal. The primary pathophysiologic abnormality seen in DHF and DSS is an acute increase in vascular permeability that leads to leakage of plasma into the extravascular compartment, resulting in hemoconcentration, hypoproteinemia, and decreased blood pressure *(420)*. Plasma volume studies have shown a reduction of more than 20% in severe cases.

One theory explaining the pathogenic changes that take place in DHF and DSS is known as the secondary-infection or immune-enhancement hypothesis *(404, 416, 417)*. Upon infection, the host immune response will produce specific antibodies against the particular serotype's surface proteins that prevent the virus from binding to the macrophages (the target cell that dengue viruses infect) and gaining entry. However, if another dengue serotype infects the same individual, the virus will activate the immune system to respond to the attack as if it had been provoked by the first serotype; the host antibodies will bind to the viral surface proteins but will not inactivate the virus. Moreover, the immune response will attract numerous macrophages, which the new dengue serotype will proceed to infect because it has not been inactivated. The body will also release cytokines that cause the endothelial tissue to become permeable, resulting in hemorrhagic fever and fluid loss from the blood vessels. Thus, it is hypothesized that prior infection, through a process known as antibody-dependent enhancement (ADE), enhances the infection and replication of dengue virus in cells of the mononuclear cell lineage *(417, 421–423)*. As a result, the patient's condition progresses to dengue shock syndrome.

Another hypothesis assumes that dengue viruses, like all animal viruses, vary and change genetically as a result of selection pressures as they replicate in humans and or mosquitoes and that there are some virus strains that have greater epidemic potential *(405, 424, 425)*. Phenotypic expression of genetic changes in the virus genome may include increased virus replication and viremia, severity of disease (virulence), and epidemic potential *(403)*.

Immunopathogenesis of DHF/DSS

During the past four decades, seroepidemiologic studies in areas endemic for DHF/DSS have provided growing evidence that the risk of severe disease has been significantly higher in secondary dengue infections and suggest that DHF and DSS have an immunologic basis *(426)*. After re-infection with a dengue virus of different serotype, severe disease is linked to high levels of antibody-enhanced viral replication early in the illness, which is followed by a cascade of memory T-cell activation and a host of inflammatory cytokines and other chemical mediators. These compounds are released primarily from T-cells, monocytes/macrophages, and endothelial cells to ultimately cause an increase in vascular permeability. These immunologic events have underscored the fact that the DHF/DSS pathogenesis is a complex, multifactorial process involving cocirculation of various dengue serotypes and the interplay of host and viral factors that influence the disease severity *(426)*.

23.13.3 Omsk Hemorrhagic Fever

The Omsk hemorrhagic fever virus (OHFV) is a member of the tick-borne encephalitis (TBE) antigenic complex of the genus Flavivirus of the family Flaviviridae. The enveloped virions are spherical, about 45 nm in diameter, with a single-stranded, positive-sense RNA genome.

OHFV is native to the western regions of Siberia and was first isolated in 1947 from the blood of a patient with

hemorrhagic fever during an epidemic in Omsk and the regions of Novosibirsk, Kurgan, and Tyumen. The virus is carried by ticks of the genera *Dermacentor* (*D. reticulates* and *D. marginatus*) and *Ixodes* (*I. persulcatus*) that remain infective for life. The animal hosts are rodents and the muskrat (*Ondatra zibethica*), and the narrow-skulled (*Microtus gregalis*) and water (*Arvicola terestris*) voles *(427)*.

The virus is susceptible to drying and heating (56°C for 30 minutes) but can survive in water and may be transmitted to humans through contaminated water. In addition to physical inactivation, OHFV is also sensitive to 70% ethanol, 1% sodium hypochlorite, and 2% glutaraldehyde.

Transmission. OHFV is transmitted to humans by the bite of an infected tick or can be caught from infected muskrats and voles (blood, feces, or urine). Rodents are infected with OHFV from the bite of an infected tick. Data suggest direct transmission (zoonosis) from both muskrats and voles. There is no human-to-human transmission reported. Aerosol infections have occurred in the laboratory. OHFV can also be transmitted through the milk of infected goats or sheep and has been isolated from aquatic animals.

All ages and both genders are susceptible to infection, and seasonal occurrence in each area coincides with vector activity.

23.13.3.1 Sequence of the Envelope Glycoprotein of the OHFV

The OHFV is of particular interest because, in contrast with most of other TBE viruses, it produces a hemorrhagic disease closely resembling but milder than that caused by the Kyasanur Forest disease virus, another highly pathogenic member of the group I serocomplex (see Section 23.13.3.2).

To shed light on this intriguing phenomenon, the gene encoding the envelope glycoprotein of the OHFV was cloned and sequenced *(428)*. A freeze-dried preparation of infected suckling mouse brain suspension was used as the source material for the viral RNA. The derived cDNA was amplified using PCR, and the cloned DNA was sequenced by dideoxynucleotide sequencing. Alignment of the OHFV sequence with those of other known tick-borne flaviviruses showed that they shared *N*-glycosylation sites, cysteine residues, the fusion peptide, and the hexapeptide (EHLPTA; amino acids 207 to 212) that identifies tick-borne flaviviruses. Though OHFV was distinguishable from the other viruses, it shared the closest amino acid identity (93%) with the TBE viruses. A sequence of three amino acids (AQN; amino acids 232 to 234), which had previously been known to be specific for the TBE viruses, was altered to MVG (amino acids 232 to 234).

In addition to the amino acid substitutions described above, the OHFV also showed several other unique amino acid substitutions in positions that may have significance for its pathogenesis. For example, the substitution of an amino acid in OHFV at position 67 exactly coincided with that of an escape mutant of TBE virus *(429)* and this is the position of the *N*-glycosylation site in the dengue virus. Furthermore, the amino acids 271 to 282 encompassed a cluster of amino acid changes in OHFV, and an amino acid codon change within this region has been identified in a Japanese encephalitis virus neutralization-resistant mutant *(430)*. Moreover, the vaccine strain of yellow fever virus also showed two amino acid codon substitutions in this region *(431)* compared with the similar regions in most other flaviviruses. These and other observations have strongly implied that changes in several domains within the viral envelope glycoprotein may be required for altering the pathogenic characteristics of the OHFV—that is, there is no single motif that will determine its hemorrhagic pathogenicity, but rather a combination of substitutions of the types described above may be required for stabilizing attenuation *(428)*.

23.13.3.2 Omsk Hemorrhagic Fever: Human Disease

The incubation period after initial infection is usually between 3 and 8 days. The illness begins with a sudden onset of fever, chills, headache, pain in both the lower and upper extremities, and severe prostration. A papulovascular rash on the soft palate, cervical lymphadenopathy, and conjunctival suffusion are usually present. Patients may also experience abnormally low blood pressure. CNS abnormalities (signs of encephalitis) typically develop 1 to 2 weeks after the onset of disease.

Severe cases of illness present with hemorrhages but no cutaneous rash. Other forms of hemorrhages include epistaxis (nosebleed), and gastrointestinal and uterine bleeding. The lungs may also be affected.

The Omsk hemorrhagic fever is a biphasic illness (i.e., after the initial appearance of symptoms, there may be a brief period of recovery before new symptoms appear). The second phase usually will develop after 1 to 2 weeks and will affect the central nervous system.

Laboratory findings including leukopenia and thrombocytopenia are indicative of viral hemorrhagic fever. Other diagnostic tests include detection of antigens or antibody to the virus in the blood—immunoassays distinguishing OHFV from related viruses (which are available in Russia), and ELISA (enzyme-linked immunosorbent serologic assay).

Complications after recovery frequently include hearing loss, hair loss, and behavioral or psychological difficulties associated with neurologic conditions.

Previous infection leads to immunity.

The mortality rate of Omsk hemorrhagic fever is estimated to be between 1% and 10%.

Treatment. There is no specific treatment (e.g., antiviral drugs) for Omsk hemorrhagic fever. A formalized mouse-brain vaccine is in use but is not available commercially. Supportive therapy is important. Replacement therapy should be considered only in severe cases of hemorrhages, and usual precautions should be taken for patients with bleeding disorders. Fluid infusion to deal with dehydration is often counterproductive but can be supportive if accompanied by careful monitoring of serum electrolytes.

23.13.4 Kyasanur Forest Disease

The Kyasanur Forest disease (KFD) virus was first recognized in 1957 as a febrile illness in the Shimoga district of Karnataka State in India *(432)*. Preceding the human epidemic, a large number of sick and dead monkeys had been noticed in the nearby forest area. The virus has been isolated from dead monkeys, sick patients, and the vector tick species *Haemaphysalis spinigera*. Since the first case, there has been a centripetal expansion of the affected area, presumably from an altered ecosystem resulting from deforestation and changing agricultural practices *(433)*. Recently, a virus very similar to KFD virus (KFDV) was discovered in Saudi Arabia.

The KFDV is susceptible to disinfectants (70% ethanol, 1% sodium hypochlorite, and 2% glutaraldehyde), as well as to heating at 56°C for 30 minutes, and to freezing.

The main hosts of KFDV are small rodents, but shrews, bats, and monkeys may also carry the virus (http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/kyasanur.htm).

Transmission. KFDV is transmitted by the bite of an infected tick (*H. spinigera*), especially nymphal ticks. Humans can get the disease from a tick bite or by contact with an infected animal (zoonosis), such as sick or recently dead monkey. Larger animals such as goats, cows, and sheep may become infected with KFDV, but they do not have a role in the transmission of the disease. Furthermore, there is no evidence that the infection is transmitted through unpasteurized milk of any of these animals.

23.13.4.1 KFDV: Structural Protein Gene Sequence

KFDVs have been isolated from monkeys, humans, and ticks from different areas and at different times, and they were found to be antigenically identical *(434)*. Although antigenic relationships have been demonstrated between the KFDV and other tick-borne flaviviruses, significant differences have been found in the neutralization indices between KFD, European tick-borne encephalitis (TBE), and the Russian TBE (formerly known as the Russian spring-summer encephalitis virus) viruses *(435)*. The antigenic discreteness of KFDV was further demonstrated when it was observed that mice immunized with inactivated KFDV showed no resistance to challenge with the Russian TBE *(436)*.

However, the relationships of KFDV with other members of the tick-borne flaviviruses at molecular level is less clear. The sequence of the structural protein genes of the KFDV has been defined using conserved primers in a PCR assay *(437)*. Data have shown that the KFDV is a distinct member of the tick-borne antigenic complex with characteristic protease cleavage sites, fusion peptides, signal sequences, and hydrophobic transmembrane domains. Among its structural proteins, the KFDV E glycoprotein exhibited maximum similarity (77.4% to 81.3%) to tick-borne flaviviruses.

The C protein was highly basic (21.5% lysine and arginine residues) and similar to other flaviviruses. The C protein is believed to be important in the interaction with RNA during the formation of viral nucleocapsids. Furthermore, the KFDV showed the sequence AKG after the first M (membrane protein), which was unique among all tick-borne flavivirus sequences *(437)*.

The E glycoprotein represents the most important functional component of the KFDV by inducing inhibition of hemagglutination and by neutralizing protective antibodies during the course of natural infection or immunization. It is thought to be one of the major determinants of virulence or attenuation of the virus.

Although the tick-borne flavivirus-specific hexapeptide genetic marker EHLPTA showed a T $\rightarrow$ K substitution (at residues 207 to 212) unique to the KFDV, the alignment of its amino acid sequence with those of other known tick-borne flaviviruses revealed many conserved regions confirming its identity as a member of the TBE antigenic complex *(437)*.

The previous belief that the KFDV was a variant of the Omsk hemorrhagic fever virus (see Section 23.13.3) introduced from Siberia is questionable because the sequence homology of the E glycoprotein of the two viruses was no greater than that of other members of the group 1 serocomplex *(437)*.

23.13.4.2 KFD: Human Disease

After an incubation period of 3 to 8 days, the symptoms of the KFD begin suddenly with fever, headache, severe muscle pain, cough, dehydration, gastrointestinal symptoms, and bleeding abnormalities. Patients may also experience abnormally low blood pressure and low platelet, red blood cell, and white blood cell counts. Relative bradycardia is frequently present, along with inflammation of the conjunctivae (http://www.cdc.gov/ncidod/dvrd/spb/mnpages/dispages/kyasanur.htm) *(438)*. After 1 to 2 weeks of symptoms, some patients recover without complication.

However, in most patients the illness is biphasic, and at the end of the second week, patients begin to experience a

second wave of symptoms, including fever and signs of CNS disorders (encephalitis).

It is estimated that there are approximately 400 to 500 cases of KFD annually in India with a mortality rate ranging between 3% and 5%. The hemorrhagic fatality rate is 2% to 50%. A small number of patients will develop coma or bronchopneumonia before death.

Diagnosis of the disease is made by isolating the virus from blood or by serologic testing using the ELISA assay.

Treatment. There is no specific treatment for KFD, but supportive therapy is highly important, including hydration and treatment for bleeding disorders.

23.14 Protein Toxin Weapons

The term *toxin weapon* has been used to describe poisons typically of natural origin and easily accessible by modern chemical synthetic methods. When used in the battlefield or as bioterrorist agents, they cause death or severe incapacitation at relatively low concentrations *(439, 440)*.

Recently, several factors have increased the importance of these agents as biological weapons, namely *(439)*:

(i) The progress in biotechnology that made the large-scale production and purification of protein toxins more feasible.
(ii) New molecular biology techniques such as polymerase chain reaction (PCR) that enable the identification, isolation, and comparison of extended families of previously less well known natural toxins.
(iii) New developments in gene manipulation and microbiology that have significantly expanded the accessible delivery vehicles for proteins to include, for example, natural or genetically modified bacteria and engineered viruses as sources for novel protein toxins.
(iv) The ability for aggressors to use toxins as a tactical weapon to strike at the enemy in a controlled manner that is difficult or impossible with infectious agents, for example by selective contamination of key terrain or high-value targets. Aerosolized protein toxins can be used both as lethal or severe incapacitating agents, greatly complicating medical care and logistical systems.

However, the absence of replicating biological delivery systems will markedly reduce the usefulness of protein toxins as direct mass-casualty biological weapons. Further drawbacks include the facts that:

(i) Proteins are not volatile and generally do not persist long in the environment.
(ii) Simple, physical protection offers an effective natural defense against foreign proteins.
(iii) Relatively sophisticated research, development, testing, and evaluation is required to establish conclusively that each specific protein toxin is a viable open-air aerosol weapon.

23.14.1 *Clostridium botulinum* Neurotoxins

Botulism is caused by a family of potent neurotoxins (*Clostridium botulinum* neurotoxins; BoNTs) produced by *Clostridium botulinum* bacteria from one of at least seven different serotypes designated as BoNT/A through/G *(441)*. Four of these serotypes (BoNT/A,/B,/E, and to a lesser degree/F) are significant as human poisons through contaminated food, wound infection, or infant botulism *(440)*. Although rare, the extreme toxicity of BoNT qualifies it as a potent biological weapon. Thus, after internalized, BoNT may cause fatal paralysis in animals at nonagram/kilogram levels *(442)*.

During the past decade, the three-dimensional structures of holotoxins or isolated toxin domains of *Clostridium* bacteria have been solved, rendering a clearer picture of their functions and toxicities *(443–446)*. Like the closely related tetanus neurotoxin (TeNT) *(443)*, the BoNT proteins are disulfide-bonded heterodimers consisting of an approximately 50-kDa zinc metalloproteinase "light chain" and an approximately 100-kDa receptor-binding "heavy chain" (Hc). The Hc has been subdivided structurally and functionally into a *C*-terminal domain that binds the toxin to gangliosides and other receptors on the surface of the peripheral cholinergic neurons (so-called Hc domain), and the *N*-terminal domain, which is believed to enhance cell binding and translocation of the catalytic light chain across the vascular membrane. In addition, the BoNT naturally is associated with a number of nontoxic "accessory proteins," some of which may stabilize the toxins *in vivo (439)*.

The mechanism by which BoNT traverses the neuron cell membranes is not completely understood, but once inside the neuron, the catalytic light chain subunit of BoNT acts as a selective zinc metalloproteinase to cleave essential polypeptide components of the so-called *SNARE complex* required for normal neurotransmitter release or membrane fusion. The exact mechanism by which the soluble *N*-ethyl maleimide–sensitive factor attachment protein receptors (SNARE) complex mediates vesicle fusion or release of neurotransmitter acetylcholine (ACh) into the synaptic cleft remains controversial; however, it is clear that the integrity of the complex is critical for normal cholinergic nerve transmission *(447, 448)*.

Clinical Manifestations of BoNT Intoxication. By disrupting ACh exocytosis at the peripheral neuromuscular junction,

BoNT causes cholinergic autonomic nervous system dysfunction in affected patients. The signs and symptoms of BoNT intoxication, which typically manifest 12 to 36 hours after toxin exposure, include generalized weakness, lassitude, and dizziness. There may be decreased salivation and dry mouth or sore throat. Furthermore, motor symptoms will reflect cranial nerve dysfunction, including dysarthria, dysphonia, and dysphagia, followed by symmetric descending and progressive muscle paralysis *(449)*. Without adequate supportive care, death may occur abruptly as a result of respiratory failure *(439)*.

Current medical treatment for BoNT intoxication is likely to involve prolonged life-support for incapacitated survivors, including the continual use of mechanical ventilation *(450)*.

23.14.1.1 BoNT Vaccines

It is known that the tetanus neurotoxin (TeNT) Hc fragments could compete for neuron binding and thereby antagonize the neuromuscular blocking properties of native TeNT and, to a lesser degree, BoNT *(451, 452)*. These observation led to the development of a vaccine candidate for BoNT/A based on the recombinant Hc once the BoNT gene was cloned and expressed. Subsequent epitope mapping of BoNT/A identified two specific polypeptides, both from Hc ($H_{455–661}$ and $H_{1150–1289}$), which were capable of protecting mice from supralethal challenge with the botulinum neurotoxin *(453)*.

Further studies at the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) have led to the development of recombinant BoNT Hc vaccine candidates for BoNT/A,/B, and/F, which conferred protection in mice against supralethal challenge with the botulinum neurotoxin *(454–458)*. This approach was recently extended to include BoNT Hc fragments from BoNT/C and/D *(459)*.

Unlike the BoNT toxoids, the recombinant Hc vaccine candidates did not require treatment with denaturants and were not susceptible to reversion of catalytic activity *(439)*.

Although apparently safe and effective, the inherent limitation of the recombinant Hc fragment vaccines has been their lack of cross-reactivity among the BoNT serotypes; a separate Hc fragment immunogen would be required for each BoNT serotype and perhaps for some different strains of each BoNT serotype.

Alternative Delivery of BoNT Vaccines. Several alternative vaccine-delivery systems for recombinant BoNT Hc immunogens have been explored recently in animal models, including inhalation and oral vaccine delivery, as well as the use of self-replicating RNA virus or DNA-based vectors *(460–463)*. Proof of concept for the use of inactivated holotoxin as an oral immunogen has also been reported *(464, 465)*. However, it remains unproven whether these experimental delivery approaches offer any practical advantage compared with traditional intramuscular vaccination *(439)*.

23.14.1.2 Immunotherapeutics

The potential of BoNT as a mass casualty biological weapon, coupled with the high cost and logistical burden of symptomatic medical treatment, has prompted an increased effort to develop selective and cost-effective BoNT therapeutics *(439)*.

Animal and human studies have suggested that the presence of preformed, neutralizing antibodies in the serum to bind and eliminate the BoNT before it reaches the target cells can prevent and/or reduce intoxication. Thus, several antitoxin preparations for human use have been developed, including a "trivalent" (serotypes/A,/B, and/E) equine antitoxin product, as well as a monovalent BoNT/E antitoxin *(439)*. In addition, an experimental despeciated equine heptavalent (serotypes/A to/G) product has been developed at USAMRIID and currently is administered under Investigational New Drug (IND) protocol for limited use *(439)*.

Several studies resulted in the production of a human *botulism immune globulin (BIG)* antiserum from different pools of plasma obtained from human donors who had been previously vaccinated with the pentavalent toxoid (PBT) vaccine. Subsequently, a human antitoxin (BIG-IV) was developed and distributed for treating infant botulism under FDA-authorized IND protocol *(466)*. Intravenous administration of BIG significantly reduced the hospital stay of infants diagnosed with botulism. However, it is still not clear to what extent the success of BIG with infant botulism will also apply to treating patients exposed to BoNT by aerosol *(439)*.

23.14.2 *Staphylococcus aureus Enterotoxins*

Whereas BoNT achieves its potency by dampening an amplified extracellular signal of nerve cells via enzymatic catalysis, the *Staphylococcus aureus* enterotoxins (SEs) act by inappropriately amplifying an extracellular signal of key immune cells *(439)*.

The SE belong to an extended family of stable 23- to 29-kDa protein toxins that includes SE serotypes/A,/B,/C1,/C2,/C3,/D,/E, and/H, and streptococcal pyrogenic exotoxin serotypes/A to/C,/F to/H, and/J, as well as toxic shock syndrome toxin (TSST-1) *(467)*. Based on their common ability to cause severe illness in animals by inducing a physiologic overreaction of the host-immune response, these toxins have been categorized collectively as *superantigens (SAgs) (439)*. SAgs are a major cause of human poisoning

and contribute significantly to opportunistic bacterial infections in hospital patients.

In the context of biological weapons, the most important SAg is SE serotype/B (SE/B). SE/B is a two-domain, α/β-protein that contains discrete binding sites for the major histocompatibility complex (MHC) class II molecule and the Vβ regions of T-cell antigen receptors (TCRs) *(468–470)*. By binding to these two receptor molecules, and perhaps through other cell-surface interactions, SE/B is able to activate both antigen-presenting cells and a relatively large number of T lymphocytes to cause the release of pyrogenic cytokines, chemokines, and other proinflammatory molecules *(439)*.

Whereas the more common forms of SAg food poisoning can be managed with routine supportive care, SE/B is a formidable aerosol threat because of its high potency and stability. It is estimated that SE/B may produce human incapacitation and death at levels as low as 0.03 and 1.5 μg, respectively *(439)*.

23.14.2.1 SE Vaccines

SE Toxoid Vaccines. During the mid-1960s, it was shown that SE/B can be isolated from bacterial culture supernatants in highly purified form and then inactivated with neutral formaldehyde solution to produce an effective toxoid vaccine *(471)*. However, the requirement of active toxin production as starting material, the possibility of toxoid reversion to yield active SE/B toxin, as well as minor reactogenicity associated with formaldehyde-inactivated vaccines, has prompted research studies directed at the development of improved SE/B vaccines.

Engineered SE Vaccines. Comparative structural and biochemical studies have focused on the development of nontoxic, recombinant immunogens capable of eliciting a protective immune response against multiple SAg toxins. One such initiative was to inactivate SE by modifying three structural regions of the toxin that were involved in HLA-DR1 binding: (i) a polar pocket created by three β-strand elements of the β-barrel domain of the toxin; (ii) a hydrophobic reverse turn; and (iii) a disulfide-bonded loop *(468,472–474)*. By combining substitutions in each of these three structural regions of SE/B (Tyr89Ala, Leu45Arg, and Tyr94Ala) within a single immunogen, a recombinant candidate (rSE/Bv) was produced that was lacking detectable SAg activity. An analogous recombinant immunogen was subsequently developed for SE type A (SE/A) by introducing comparable substitutions (Asp70Arg, Leu48Arg, and Tyr92Ala) *(439)*.

The rSE/Bv vaccine candidate was tested in mouse and non-human primate models and was found to elicit high antibody titers, and the vaccinated mice survived supralethal challenges with SE/B toxin. In contrast with the natural toxin, rSE/Bv showed no evidence of toxic SAg activity *(439)*.

23.14.3 *Clostridium perfringens*

Clostridium perfringens is an anaerobic Gram-positive spore-forming bacillus that produces at least 15 toxins *(475)*. The toxins of this microorganism are highly toxic phospholipases.

The spores germinate after warming slowly at ambient temperature. The natural reservoir of the microorganism is soil and the gastrointestinal tract of healthy persons and animals.

The virulence factor of *C. perfringens* is responsible for various disease, including gas gangrene, food poisoning, necrotic enteritis, and enterotoxemia *(476)*. Gas gangrene is typically the result of contamination of a wound by spores of *C. perfringens*. Unlike *B. anthracis* spores, there is no apparent disease after inhalation of *C. perfringens* spores. There have been rare case reports of pulmonary infections associated with *C. perfringens (477, 478)*.

Whereas aerosol challenges with*C. perfringens* toxin have been reported to produce lethal pulmonary disease in laboratory animals, there is no established threat from aerosol exposure and inhalation of these toxins in healthy individuals *(475)*.

23.14.4 Ricin Toxin

Ricin is a disulfide-bonded heterodimeric toxin found in the seeds of the castor bean plant (*Ricinus communis*). It has been recognized as a potential toxin weapon since World War I *(439)*. Although less lethal than BoNT and SE, at sublethal doses it can cause incapacitation by pulmonary damage. Moreover, ricin is readily available because the castor bean plant is cultivated worldwide, and the toxin is easily extracted from common by-products of the seeds *(479, 480)*.

The name *ricin* was given to the toxin by R. Stillmark in 1888 when he tested the beans' extract on red blood cells and saw them agglutinate *(481)*. Now it is known that the agglutination was caused by another toxin also present in the extract, called RCA (*Ricinus communis* agglutinin). The difference between these two toxins is that ricin is a potent cytotoxin but a weak hemagglutinin, whereas RCA is a weak cytotoxin but a powerful hemagglutinin. Poisoning by ingestion of the castor bean is due to ricin, not RCA, because the latter does not penetrate the intestinal wall and does not affect the red blood cells unless given intravenously. If injected into the blood, RCA will cause the red blood cells to agglutinate and burst by hemolysis.

History and Military and Medical Significance. In ancient Egypt, *Ricinus communis* was cultivated for its oil's lubricating and laxative effects. Both castor bean oil and whole seeds have also been used in various regions of the world in the treatment of other diseases. During World Wars I and II, the lubricating oil was used in the aircraft industry until synthetic oils replaced the castor oil.

During the Cold War, ricin was used in the highly publicized assassination of the Bulgarian dissident Georgi Markov in 1978. He was killed with ricin contained in a pellet placed in an umbrella tip. Mr. Markov was stabbed with the umbrella while waiting for a bus on a London street *(479)*.

Any attack using ricin is likely to be on a small scale. Experts have estimated that 4 tons would be needed to affect 100 km^2, which would make ricin more a tool for assassination than a weapon of mass destruction. Ricin could also be used to contaminate food or water or by leaving it on door handles in busy buildings, with the aim of poisoning and spreading panic.

Recently, with the advent of new immunotherapeutic approaches, ricin has been studied as a component of anti-tumor regimens known as immunotoxins, specifically in chimeric toxins. For example, the native ricin or just its A-chain is conjugated to tumor cell–specific monoclonal antibodies (technically, to other ligands that target the active component of the toxin to tumor cells for selective killing). Some of these conjugates have undergone Phases I and II clinical trials as anticancer agents *(482, 483)*. Although the results have shown promise, two factors appear to limit ricin's immunoefficacy: the lack of specificity of the antibody, and the significant immunogenicity of the toxin moiety, which would result in relatively rapid onset of refractory immunity to the therapeutic agent *(484, 485)*.

When ingested, one to three ricin seeds may be fatal to a child; two to four may be poisonous to an adult, and eight may be fatal. A fatal ingested dose is about 1 mg/kg. Because the alimentary tract will destroy a significant amount of ricin because of its poor oral absorption from the gastrointestinal tract, it is much more potent when administered parenterally—a dose of 2 millionth of the body weight may prove fatal *(486)*.

Molecular Structure. Ricin is a heterodimeric ribosome-inhibiting protein. The ribosome-inactivating 32-kDa protein called A-chain (RTA) is linked by a disulfide bond to a 34-kDa B-chain (RTB), which is a specific galactose/*N*-acetylgalactosamine-binding lectin. Both chains are glycoproteins containing mannose-rich carbohydrate groups; the reticuloendothelial cells have mannose receptors. To confer toxicity, the A- and B-chains must be associated.

The ricin toxin has been crystallized, and its crystal structure was determined by x-ray crystallography to 2.5 Å *(487)*.

Ricin Uptake. Having bound diffusely to the cell membrane, ricin is internalized. The part of ricin bound to the cell surface undergoes receptor-mediated endocytosis. The ricin is taken up in uncoated pits, and pits and vesicles showing the characteristic clathrin coat. Smooth pits and/or large smooth invaginations may also play a part in the ricin uptake. The latter is not nearly as fast as the uptake of molecules such as LDL and transferrin, which use similar mechanisms, and thus there is a lag time between the administration of toxin and development of toxic effect.

The toxin molecule is then internalized to the vacuolar and tubo-vesicular portions of the endosomal system, where most of it remains bound to the plasma membrane, despite the prevailing acidic conditions.

Ricin is transported retrograde via the Golgi apparatus to the *endoplasmic reticulum (ER)*. For the A-chain subunit to reach its target ribosome site and cause toxic effects in the cell, it must first enter the cytosol, which it does through the ER using the so-called *ERAD (ER-associated protein degradation) pathway*. The ERAD pathway is associated with transportation of misfolded proteins to the proteosome. Ubiquitination is an important part of ERAD and may also be important in the ricin retrograde translocation. Once in the cytosol, at least some of the ricin must avoid proteasome degradation so that it can kill the cell. It is possible that ricin may be inefficiently ubiquitinylated due to a low lysine content of its A-chain. It may also be possible that the A-chain has learned to "look" like a misfolded protein in the ER, and once it is exported to the cytosol it will appear as a properly folded protein, thereby avoiding proteasome degradation.

Some ricin may reach the cytosol directly from the endosomes through endosome degradation.

Mechanism of Action. Like many other structurally and functionally related cytotoxic proteins from a variety of plants, ricin inhibits the protein synthesis by specifically and irreversibly inactivating the eukaryotic ribosomes. Typically, these ribosome-inactivating proteins (RIPs) are *N*-glycosylated *(488)*.

Ribosomes are complex structures, consisting of protein and nucleic acid (rRNA) components. Structurally, they have two subunits, a large subunit that contains an rRNA fragment (known as the 60S fragment), and a smaller subunit. The 60S fragment is made of several pieces of RNA, one of which is the 28S rRNA. It is believed that the RNA components are most important in the protein chain elongation catalysis. The ribosomes are responsible for protein synthesis from mRNA and amino acid subunits linked to tRNA.

The ricin A-chain catalytically and irreversibly inactivates the 60S large ribosomal subunit by binding and depurinating a specific adenine (at base A_{4324}) of the 28S rRNA fragment of the 60S RNA chain. The target adenine is a specific RNA sequence that contains the unusual tetranucleotide

loop, GAGA. Depurination occurs at base A_{4324}, which is part of the GAGA loop.

The adenine ring of the ribosome becomes sandwiched between two tyrosine rings in the catalytic cleft of the enzyme (A-chain) and is hydrolyzed by the enzyme's *N*-glycosidase action. This change does not directly cause the hydrolysis of the RNA chain but renders the phosphodiester bonds surrounding the altered base highly susceptible to hydrolysis. This affects the binding of elongation factors to the ribosome and thus halts the protein synthesis; it requires no energy or cofactors.

Inactivation/Decontamination. Ricin can be inactivated by heat: 80°C for 10 minutes or 50°C for approximately 1 hour at pH 7.8. An 0.1% sodium hypochlorite solution is thought to be effective, although some have recommended using a stronger 0.5% sodium hypochlorite solution.

23.14.4.1 Clinical Manifestations of Ricin Intoxication

After ingestion, the incubation period is a few hours to a few days. After inhalation, the incubation period appears to be less than 8 hours. Humans who were accidentally exposed to sublethal doses of ricin would develop symptoms in 4 to 8 hours *(488)*.

Diagnosis. The diagnosis of ricin poisoning is usually based on clinical signs. Ricin can sometimes be detected in serum or respiratory secretions by ELISA and in tissues by immunohistochemistry. PCR assays can often find castor bean DNA in ricin preparations.

Ricin is very immunogenic, and serology can be useful for a retrospective diagnosis. ELISA and chemiluminescence tests are available.

Clinical Symptoms After Ingestion. Natural infections typically occur after ingestion. Onset of symptoms is usually within 4 to 6 hours but may be as late as 10 hours. The initial signs are nonspecific and may include colicky abdominal pain *(488)*. The ingested toxin usually manifests with severe gastrointestinal symptoms, including abdominal pain, diarrhea, fever, nausea, vomiting, incoordination, drowsiness, and hematuria. The fluid loss can lead to dehydration, particularly in children. Vascular collapse and death occur quickly, but most patients who survive for 3 to 5 days will recover.

Clinical Symptoms After Aerosol Inhalation. After aerosol exposure, the symptoms appear acutely with clinical signs that include fever, tightness of the chest, cough, dyspnea, nausea, and arthralgia. In sublethal doses, profuse sweating will occur several hours later, followed by recovery. Lethal human exposures by aerosol have not been reported. Based on studies in laboratory animals, airway necrosis and pulmonary edema would probably develop within 24 hours. Severe respiratory distress and death from respiratory complications and circulatory collapse would be expected to occur within 36 to 72 hours. The symptoms would likely include dyspnea, cyanosis, and hypotension.

Clinical Symptoms After Parenteral Intoxication: The Case of Georgi Markov. In the assassination of Georgi Markov in 1978, the ricin was placed in a pellet hidden in an umbrella tip *(480)*. The pellet, containing about 500 μg ricin, when injected into his body during the attack resulted in almost immediate local pain, then a feeling of weakness within 5 hours. Fifteen to 24 hours later, the victim had a high temperature, nausea, and vomiting. Thirty-six hours after the attack, he was admitted to the hospital feeling very ill, presenting with fever and tachycardia, but normal blood pressure. The lymph nodes of the affected groin were swollen and a 6-cm-diameter area of induration and inflammation was observed at the injection site on his thigh. Just over 2 days after the attack, Mr. Markov became suddenly hypotensive and tachycardic; his pulse rate was 160. The white blood count was $26,300/\text{mm}^3$. Early on the third day after the attack, the victim became anuric and began vomiting blood. An electrocardiogram demonstrated a complete atrioventricular conduction block. Mr. Markov died shortly thereafter; at the time of his death, his white blood cell count was $33,200/\text{mm}^3$ *(480)*.

Intramuscular or subcutaneous injection of toxin doses, as in the case of Mr. Markov, would result in severe local lymphoid necrosis, gastrointestinal hemorrhage, liver necrosis, diffuse nephritis, and diffuse splenitis. In the case of Mr. Markov, a mild pulmonary edema was thought to be secondary to cardiac failure *(480)*. Similar data have been collected after experimental animal studies.

23.14.4.2 Treatment of Ricin Intoxication

Because no antidote exists for ricin poisoning, the most important factor is to get the ricin off or out of the body as quickly as possible and to give the victim supportive medical care to minimize the effects of the poisoning.

For oral intoxication, supportive therapy includes administering activated charcoal and intravenous fluid and replacing electrolytes. For inhalational intoxication, supportive therapy is aimed at counteracting acute pulmonary edema and respiratory distress. Symptomatic care is the only intervention currently available for the treatment of incapacitating or lethal doses of inhaled ricin *(480)*.

Animal studies have shown that either active immunization or passive prophylaxis or therapy (if the therapy is given within a few hours) is extremely effective against intravenous or intraperitoneal intoxication with ricin. On the other hand, inhalational exposure is best countered with active immunization or prophylactic administration of aerosolized specific antiricin antibody *(480)*.

Ricin Vaccines

Ricin Toxoid Vaccines. A toxoid vaccine prepared from formalin-inactivated ricin holotoxin was developed during World War II and was shown to enhance survival significantly in animals exposed to ricin *(489)*.

An improved ricin toxoid vaccine based on denatured toxin adsorbed to Alhydrogel adjuvant was developed at USAMRIID in the 1990s and has been shown to be effective at protecting rhesus monkeys against ricin toxin aerosol exposure *(439)*. However, as with earlier studies, the vaccine did not protect completely against short-term (up to 14 days postexposure) bronchiolar and interstitial pulmonary inflammation.

The general failure of toxoid vaccines to protect the respiratory tract of exposed animals from the cytotoxic effects of ricin underscores the need to develop effective recombinant vaccines and alternative vaccine-delivery systems that can elicit and enhance mucosal immune response *(490, 491)*.

Deglycosylated Ricin A-Chain Vaccine. The ricin A-chain (RTA) conjugated with tumor-specific antibodies has been used clinically in animal and human studies to target and kill tumors *(483, 484)*. These and other studies *(492)* have contributed to the development of a recombinant ricin vaccine by demonstrating convincingly that the ricin A-chain in the absence of the B-chain is much less toxic than is the whole toxin when administered parenterally to animals *(493, 494)*.

Some technical limitations have arisen regarding the use of RTA or dgRTA (chemically deglycosylated RTA) as candidates for human vaccines. Both immunogens have retained residual *N*-glycosidase activity and have shown significant aggregation during expression and purification or upon prolonged storage in solution *(439)*. To this end, recombinant vaccine candidates with active-site–specific substitutions designed to reduce the *N*-glycosidase activity of RTA without disrupting the antigenic properties of the molecule have been proposed as vaccine candidates *(492, 495–498)*. Active-site substitutions in RTA essentially eliminated the problem of residual toxicity but did not address the important manufacturing problem regarding RTA instability and aggregation *(439)*.

Engineered Ricin Vaccines. It is thought that the tendency of subunit-based RTA vaccines to self-aggregate under physiologic conditions is related to the hydrophobic domains being exposed by the absence of the natural B-chain (RTB) subunit. Starting from a theoretical analysis of the functional architecture of the toxin compared with the related single-chain ribosome-inactivating proteins (RIPs) *(498–500)*, it was hypothesized that reducing the hydrophobic surface of RTA by large-scale deletions might result in a better structural platform for presenting the neutralizing epitope than that of the parent molecule.

Furthermore, along with reduced hydrophobic surface, recombinant vaccine candidates would have to retain the surface loop which is thought to serve as a neutralizing immunologic epitope for the ricin toxin (i.e., RTA residues 97 to 106; Ref. *493*). In addition, candidates would also have to lack key amino acid residues of the RNA binding site that is essential for *N*-glycosidase toxicity. Based on experimental trials with an array of recombinant RTA candidates, it was found that immunogens based approximately on the *N*-terminal domain of RTA (residues 1 to 198) best satisfied these design criteria *(439)*.

Under physiologic conditions, polypeptides based on RTA residues 1 to 198 remained folded as seen by circular dichroism and infrared spectroscopy, were more stable thermodynamically than was RTA, and exhibited dynamic light scattering, indicating monodisperse monomers without significant aggregation. Moreover, the single-domain immunogens showed no detectable toxin activity and protected mice against supralethal exposure to ricin toxin by injection or by aerosol *(439)*. In this case, protein engineering based partly on a functional analysis of protein domains has yielded ricin vaccine candidates that were superior to traditional approaches, including inactivated holotoxin or toxin subunit vaccines containing active-site mutations *(439)*.

23.14.5 Abrin Toxin

Abrin is a potent plant toxin that has been isolated from the seeds of *Abrus precatorius* (the rosary pea). Its cell agglutinating activity was first described in 1972 *(501)*, and its biological activity is very similar to that of ricin: the A-chain of abrin inhibits protein synthesis, whereas its B-chain binds to the cell surface receptors containing terminal galactose and acts as an immunotoxin. The A-chain is not active until it is internalized by the cell.

As with ricin, the extreme toxicity of abrin and its relatively easy manner of production makes it a potential biological weapon.

Molecular Structure and Mechanism of Action. Like ricin, abrin structurally represents a heterodimer consisting of two peptide moieties (A-chain and B-chain) linked by a disulfide bridge. Ricin and abrin have a large-scale molecular similarity, with the A-chains of both toxins having a 102 conserved amino acid homology.

Abrin exists in two forms, abrin-a and abrin-b, with both containing the A- and B-chains. A disulfide bond between Cys247 of the A-chain and Cys8 of the B-chain connects the two chains.

The A-chain comprises 251 residues and is divided into three folding domains. The A-chain catalytically inactivates 60S ribosomal subunits by removing adenine from positions

4 and 324 of 28S rRNA, resulting in the inhibition of the protein synthesis.

The B-chain represents a galactose-specific lectin that facilitates the binding of abrin to the cell membranes *(502, 503)*. The B-chain of both forms of abrin consists of 268 amino acid residues and shares 256 identical residues *(504)*. A comparison of the B-chains of abrin-a and abrin-b with that of ricin has shown that 60% of the residues of abrin's B-chain were identical to those of the B-chain of ricin. In addition, two saccharide-binding sites in the ricin B-chain identified by crystallographic studies were highly conserved in the abrin B-chain *(504)*.

The mechanism of action of abrin is identical to that of ricin, but the toxicity of abrin in mice was 75 times that of ricin (0.04 μg/kg for abrin compared with 3.0 μg/kg for ricin).

The diagnosis, clinical features, treatment, protection, and prophylaxis of abrin intoxication are the same as those of ricin *(505)*.

23.14.6 Trichothecene

Trichothecene (T-2 toxin) is a mycotoxin antibiotic produced by the fungus *Trichothecium roseum*. Though its acute toxicity by inhalation is similar to that of the poison blister gas lewisite [dichloro(2-chlorovinyl)arsine] (ca. 10^3 mg·min/m^3), the T-2 toxin is about 10 times more potent than liquid mustard [bis-(2-chloroethyl)sulfide] with dermal exposure *(506)*.

The trichothecenes are considered primarily blister agents that cause severe skin and eye irritation at low exposure doses. Subacute exposure reduces the host's resistance to bacterial or parasitic infections.

In general, the mycotoxins are not considered to be an established threat by aerosol exposure *(507)*.

23.15 Arthropod-Borne Viral Fever and Arthropathy

The arthropod-borne alphaviruses constitute an important genus of the Togaviridae family. Their ecologic maintenance is passage from mosquito to vertebrate to mosquito. The great majority of the Alphavirus-associated human infections are either subclinical or would result in a transient and only temporarily incapacitating febrile illness *(508)*. However, a small but important fraction of these infections will proceed with either viral entry into the central nervous system causing viral encephalitis or viral-associated fever and acute arthropathy, which is caused mainly by the chikungunya virus. Other togaviruses, including the O'nyong-nyong (ONN), Igbo Ora, Mayaro, and Ross River viruses, as well as some strains of Sindbis (SIN) virus, have been associated with a similar syndrome. The togavirus rubella is also arthritogenic.

23.15.1 Chikungunya Virus

Originally, the chikungunya virus (CHIKV) (also known as the Buggy Creek virus) was probably an infection of primates in the forests and savannahs of Africa, maintained by the sylvatic *Aedes* mosquito, as it continues to be today in transmission mainly between mosquitoes and monkeys *(508)*. However, today the chikungunya virus is also the etiologic agent of *Ae. aegyptii*–transmitted urban epidemics in Africa, the Indian Ocean islands, and Asia, causing crippling arthralgia and arthritis accompanied by fever and other systemic symptoms of clinically distinctive chikungunya infection. The illness is rarely life-threatening. Nevertheless, a re-emerging disease in Africa and Asia that sometimes is clinically indistinguishable from dengue fever, chikungunya epidemics cause substantial morbidity and economic loss *(509)* (http://www.cdc.gov/travel; http://www.cdc.gov/ncidod/dvbid/Chikungunya/chikvfact.htm). In a very recent development, in September 2007 an outbreak of about 160 cases was reported in the Ravenna region of northern Italy (http://news.bbc.co.uk/2/hi/health/6981476.stm).

The word *chikungunya* is thought to be derived from the word *kungunyala* in the Swahili/Makonde language of southeastern Tanzania and northern Mozambique. It is used for both the virus and the disease and means "to walk bent over" or "to dry up or become contorted" referring to the effect of the joint pains that characterize this illness.

Genomic Studies. The complete genomic sequence of chikungunya virus (strain S27 African prototype) has been determined and the presence of an internal polyadenylation [I-poly(A)] site was confirmed within the 3′ nontranslated region (NTR) of this strain *(510)*. The complete genome was 11,805 nucleotides in length, excluding the 5′ cap nucleotide, an I-poly(A) tract and the 3′ poly(A) tail. It comprised two long open reading frames that encoded the nonstructural (2,474 amino acids) and structural proteins (1,244 amino acids). In addition, predicted secondary structures were identified within the 5′ NTR and repeated sequence elements (RSEs) within the 3′ NTR.

Amino acid sequence homologies, phylogenetic analysis of nonstructural and structural proteins, and characteristic RSEs revealed that although CHIKV is closely related to the O'nyong-nyong (ONN) virus, the chikungunya virus is in fact a distinct entity.

In another study *(511)*, the genomic sequence of a sole isolate obtained from the cerebrospinal fluid of a patient

from the Indian Ocean islands showed unique changes in nsP1 (T3011), nsP2 (Y622N), and nsP3 (E460 deletion) nonstructural proteins, not obtained from isolates from sera. In the structural protein region, two noteworthy changes (A226V and D284E) were observed in the membrane fusion glycoprotein E1. Homology three-dimensional modeling allowed mapping of these two changes to regions that are important for membrane fusion and virion assembly. Change E1-A226V was absent in the initial strains but observed in more than 90% of subsequent viral sequences from a Réunion Island strain, denoting evolutionary success—possibly due to adaptation to the mosquito *(511)*.

Epidemiology. Epidemics of fever, rash, and arthritis resembling chikungunya fever were recorded as early as 1824 in India. However, the virus was first isolated in 1952–1953 from both humans and mosquitoes during an epidemic of fever in Tanzania.

Chikungunya fever (also known as "chicken guinea") displays interesting epidemiologic profiles: major epidemics appear and disappear in a cyclical manner, usually with an interepidemic period of 7 to 8 years and sometimes as long as 20 years. Between 1960 and 1982, outbreaks of chikungunya fever were reported in Africa and Asia (Thailand, 1960s; India, 1964; Sri Lanka, 1969; Vietnam, 1975; Myanmar, 1975; and Indonesia, 1982). Recently, after an interval of more than 20 years, outbreaks of chikungunya fever have been reported in India and various Indian Ocean islands (Comoros, Mayotte, Mauritius, Réunion, Seychelles, and the Nicobar and Andaman Islands) *(512–517)* (http://www.searo. who.int/en/Section10/Section2246.htm). In some regions of India, the attack rates have reached as high as 45%, with more than 1.25 million cases reported in 2006.

Chikungunya fever, although considered to be rarely life threatening, has been shown to cause fatalities: during the 2005–2006 epidemic in Réunion Island, the number of deaths rose by 34% in February 2006 and by 25% in March 2006, compared with the same months in 2005. These increases represent a total of 170 to 180 additional deaths just for these 2 months, with many of them in patients older than 75 years of age *(518)*. A total excess of 260 deaths was reported by the French National Institute for Public Health Surveillance *(518)* for the outbreak. This would correspond with roughly a 1% case fatality ratio for estimated cases of chikungunya based on seroprevalence studies *(513)*.

The suggestion *(512)* that the recent outbreak of chikungunya fever in India *(516)* could have been caused by the same viral strain that caused the Indian Ocean islands outbreak *(517)* has been challenged, based on comparison of the E1 sequences of both strains *(511, 519)*; microheterogeneity studies have shown the presence of a single nucleotide change (T321C) found in all Indian Ocean isolates, whereas isolates from India retained the ancestral T321 nucleotide present in all other African and Asian strains *(519)*.

The clinical manifestations of chikungunya fever have to be distinguished from dengue fever, especially when co-occurrence of both fevers is observed, as was recently the case in the Maharashtra State in India. Such cases highlight the importance of strong clinical suspicion and efficient laboratory diagnosis support.

The chikungunya virus is killed by common disinfectants, moist heat, and drying.

23.15.1.1 Clinical Manifestations

The incubation period can be 3 to 12 days but is usually 3 to 7 days. There is a sudden onset of flu-like symptoms including severe headache, chills, fever (> 40°C, 104°F), joint pain, nausea, and vomiting. The joints of the extremities in particular become swollen and painful to the touch; most frequently affected joints are fingers, wrists, toes, and ankles. At the early acute stage of the disease (within 10 days of disease onset), rash may also occur *(514)*. However, "silent" CHIKV infections (with no illness) do occur, but how commonly this happens is still not known.

Typically, the clinical course of chikungunya fever involves two stages: an initial severe febrile and eruptive polyarthritis, followed by disabling peripheral rheumatism that can persist for months. Also, during the second stage there is the possibility of transitory peripheral vascular disorders. Besides the arthralgic syndrome, some patients, especially children, may present with neurologic disorders or fulminant hepatitis *(511)*.

All patients suffer from arthritis. Note: The prolonged joint pain with CHIKV is not typical of dengue fever (http://www.cdc.gov/ncidod/dvbid/Chikungunya/chikvfact.htm).

Treatment

No specific therapies are currently available. Symptomatic treatment for mitigating pain and fever using anti-inflammatory drugs along with rest usually is sufficient. While recovery from chikungunya fever is the expected outcome, convalescence is prolonged (up to a year or more), and persistent join pain may require analgesic and long-term anti-inflammatory therapy.

Vaccine. After several successful trials, a vaccine against CHIKV, a potential bioterrorist agent, was developed by the U.S. military after interest in chikungunya was aroused in the 1960s, when Thailand was overrun by simultaneous outbreaks of cholera, dengue, and chikungunya. The vaccine source was a weakened version of the Thailand CHIKV strain 15561 and was labeled TSI-GSD-218. In volunteers, it was safe, well tolerated, and highly immunogenic—8% of vaccine recipients developed chikungunya antibodies by day

28, and 85% of the recipients remained seropositive after 1 year. In 1969, an experimental vaccine using the killed chikungunya virus underwent testing in the Walter Reed Army Institute of Research (WRAIR).

23.15.2 O'nyong-nyong Virus

The O'nyong-nyong (ONN) virus is considered antigenically as a subtype of the chikungunya virus. Although similar in many other respects as well, there are highly significant differences. However, recently published data have shown that chikungunya virus and ONN virus are two distinct viruses after phylogenetic analysis (E1 protein) and serologic studies *(520)*.

The ONN virus was identified as the causative agent of a major East African epidemic in 1959 *(521, 522)*. The disease, which was referred to by the Acholi word meaning "weakening of the joints," had affected at least 2 million people. After the epidemic, ONN virus was not encountered again until its isolation from *Anopheles funestus* in 1978 *(523)*. Another *Anopheles* species, *An. gambiae*, has also been implicated as a vector of ONN.

Clinical Manifestations. ONN and CHIK viral infections result in a similar clinical syndrome *(522)*. After an incubation period thought to be at least 8 days, a sudden onset of joint manifestations occur. Rash occurred in 60% to 70% of patients an average of 4 days later, often accompanied by an improvement in symptoms *(508)*. The morbilliform eruption last for 4 to 7 days before subsiding. Fever was less common than in CHIKV infections, exceeding 101°F in only about one third of outpatients. In contrast with chikungunya disease, ONN illness is characterized by the development of markedly enlarged lymph nodes of a firm, rubbery consistency *(508)*. There are no known drugs against ONN virus, and symptomatic treatment is the therapy of choice.

23.16 NIAID Research Agenda in Biodefense

Improving the United States' defenses against bioterrorism is a key part of the U.S. government's homeland security effort. The Department of Health and Human Services (DHHS) supports activities to improve local and state public health systems, to expand existing biosurveillance efforts, and to fund research on medical countermeasures against potential bioterror agents.

NIAID is committed to accelerating development of medical tools to detect and counter the effects of a bioterrorist attack (http://www3.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/about/niaidRole.htm), including:

- Vaccines to immunize the public against diseases caused by bioterrorism agents
- Diagnostic tests to help first responders and other medical personnel rapidly detect exposure and provide treatment
- Therapies to help patients exposed to bioterrorism agents regain their health

The capability to detect and counter bioterrorism depends to a substantial degree on the state of the relevant medical science, and basic research provides the essential underpinning. The National Institutes of Health (NIH) biodefense program, spearheaded by NIAID, includes both short- and long-term research targeted at the design, development, and evaluation of the specific public health tools (diagnostics, therapies, and vaccines) needed to control a bioterrorist-caused outbreak. The generation of genome sequence information on potential bioterrorism agents is also an important component of this program.

Since 2001, NIAID has greatly accelerated its biodefense research program, launching several new initiatives to catalyze development of vaccines, therapies, and diagnostic tests. For example, in December 2004, an NIAID-funded clinical trial aimed at boosting the nation's flu vaccine supply began enrolling volunteers at four U.S. sites. In October 2004, NIAID announced $232 million in biodefense contracts for vaccine development against three potential bioterror agents: smallpox, plague, and tularemia. Also, an NIAID-funded study shed new light on how the smallpox virus attacks its victims.

Identifying Research Priorities. NIAID has set research priorities and goals for each microorganism that might be used as an agent of bioterrorism, with particular emphasis on Category A agents—those considered by the CDC to be the worst bioterror threats. NIAID's research agenda and strategic plan cover the following categories (http://www3.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/about/niaidRole.htm):

- *Basic Biology*—understanding how microorganisms and their toxic products function and cause disease
- *Immunology and Host Response*—understanding how the human immune system interacts with and defends the body against potential agents of bioterrorism
- *Design/Development/Clinical Evaluation of Therapies*
- *Vaccines*—working closely with industry to create new and improved vaccines
- *Drugs*—working closely with industry to develop and test drugs to treat diseases that may result from a biological attack
- *Genomics, Basic Research, and Infrastructure*
- *Diagnostics*—developing devices or methods to quickly and accurately diagnose diseases caused by bioterrorism agents

- *Research Resources*—establishing biosafety laboratories, databases, and other resources to help scientists conduct safe and effective biodefense research

23.16.1 Major NIAID Programs in Biodefense Research

- *Modified Vaccinia Ankara Smallpox Vaccine*. NIAID continues to support advanced development and manufacture of a modified vaccinia Ankara (MVA) vaccine for smallpox, with the intention of targeting MVA vaccine candidates that can be produced on a scale to support commercial manufacturing. Two pharmaceutical companies, Bavarian Nordic and Acambis, Inc., have played a key role in the development of MVA vaccine candidates under contracts awarded by NIAID. Clinical trials are under way in the United States to evaluate the safety and immunogenicity of MVA in healthy adults, and in the United States and Europe to determine the safety and immunogenicity of MVA in adults with atopic dermatitis and HIV. A study is under way in the United States to compare routes of administration (intradermal, intramuscular, and subcutaneous) of MVA in healthy individuals (http://www3.niaid.nih.gov/Biodefense/ Public/PDF/spox_niaid_invest.pdf).
- *Smallpox Vaccines*. In addition to work to advance MVA, NIAID continues to support the development of additional smallpox vaccines (http://www3.niaid.nih.gov/Biodefense/Public/PDF/spox_niaid_invest.pdf). Three new vaccines are currently supported: (i) a live-attenuated vaccinia virus vaccine that is attenuated by a different mechanism than that of MVA; (ii) a protein subunit vaccine that comprises four variola virus proteins that are known to be largely responsible for stimulating the protective immune response that occurs when the live virus vaccine is used, thereby limiting the adverse reactions that are usually caused by other components of the virus; and (iii) a subunit vaccine in which the homologous immunity-stimulating proteins from vaccinia are expressed in a disabled, nonreplicating Venezuelan equine encephalitis (VEE) vector.

NIAID's Vaccine Research Center (VRC) has tested MVA as an attenuated poxvirus with the potential to protect against vaccinia (the virus used to vaccinate against smallpox) or variola (the virus that causes smallpox). The vaccine has been provided by Therion Biologics Corporation as part of a collaboration agreement with the Vaccine Research Center. Two Phase I clinical trials testing MVA as a component of a safer smallpox vaccine in both vaccinia- naïve and vaccinia-immune populations have concluded.

- *Compound ST-246*. Through a SBIR grant to SIGA Technologies, Inc., NIAID is continuing to support an orally bioavailable anti-poxvirus compound (ST-246) that inhibits extracellular virus formation and protects rodents from lethal Orthopoxvirus challenge. ST-246 has been shown to be highly active against a number of orthopoxviruses both *in vitro* and in animal models. NIAID has supported the preclinical development of ST-246 and recently awarded a contract for advanced manufacturing and clinical studies *(524)*
- *Cidofovir*. Clinical protocols to assess activity of cidofovir as a treatment for complications related to smallpox vaccine have been developed in the Vaccine Treatment and Evaluation Units (VTEUs) as backup therapy after vaccine immune globulin (VIG) has been indicated. To date, no one has needed to be enrolled in this protocol.
- *Recombinant Protective Antigen Anthrax Vaccine*. NIAID is continuing its support of advanced development and production of a recombinant protective antigen (rPA) vaccine for anthrax (http://www3.niaid.nih.gov/topics/Biodefense/BiodefenseRelated/Biodefense/research/anthrax.htm).

In 2004, new contracts were awarded to two pharmaceutical companies, Vaxgen and Avecia, to build upon the companies' achievements, supporting production, testing, and evaluation of rPA vaccine; this includes two Phase II clinical trials per contract. Proof-of-concept aerosol challenge efficacy studies in rabbits and non-human primates were successfully completed for both rPA vaccine candidates, as have postexposure efficacy rabbit studies that combined antibiotic and vaccine treatments. Avecia's Phase II clinical trials were completed by the end of 2006. Both companies have validated new facilities and completed validation of processes for large-scale current Good Manufacturing Practice (cGMP) manufacturing of rPA vaccine by the end of 2006.

- *Ebola Vaccine*. Building on their previous results that showed that the prime-boost vaccination strategy produced a strong, long-lasting immune response in vaccinated non-human primates, scientists at the VRC, in collaboration with researchers at the U.S. Army Medical Research Institute for Infectious Disease (USAMRIID), have developed an Ebola vaccine. Testing was conducted to determine whether the immune response mounted against the boost component alone would be sufficient to protect monkeys against Ebola infection. Results have shown that monkeys vaccinated with only the boost survived, even those who received high doses of Ebola virus. In 2005, the VRC completed a 2-year study of the first human trial of a DNA vaccine designed to prevent the Ebola infection. The trial consisted of three vaccinations given over 3 months, and study participants were followed for 1 year. Results have demonstrated that this DNA

vaccine was safe and well tolerated with no significant adverse events, and it was capable of inducing an immune response. In another project, VRC scientists have developed a fast-acting, single-shot experimental Ebola vaccine for humans based on previous studies showing protection in monkeys. A Phase I vaccine trial in humans began in autumn 2006 (http://www3.niaid.nih.gov/news/newsreleases/2003/ebolahumantrial.htm).

Vaccines against Ebola and Marburg viruses are also under development by extramural scientists with NIAID support. A partnership grant was awarded in 2002 to develop a vaccine against Marburg virus, using an Alphavirus replicon particle technology. This vaccine is effective in protecting small animals and non-human primates against Marburg virus infection and is in advanced stages of preclinical development. Vaccines against Ebola virus are under early stages of development using a variety of approaches, which include replicon particle vaccines, virus-like particle vaccines, and recombinant protein subunit vaccines.

- *Dengue Fever*. NIAID is funding research to develop countermeasures against Dengue fever (http://www3.niaid.nih.gov/healthscience/healthtopics/dengue/). One direction is to develop a validated, fully automated, portable, point-of-care nucleic acid detection system for the rapid diagnosis of *hemorrhagic fever* syndromes caused by Category A–C biodefense viruses, including dengue. Another direction is to develop a live-attenuated tetravalent dengue (DEN) vaccine. This experimental vaccine is a mixture of the established attenuated DEN-2 vaccine strain, which has been shown to be safe and effective in human clinical trials, and chimeric viruses that express the structural genes of the other three DEN serotypes in the attenuated DEN-2 background.
- *Arenaviruses*. NIAID is supporting efforts to develop and validate new ELISA diagnostic tests to be used in clinics for the rapid detection of arenaviruses that cause viral hemorrhagic fevers in humans. These include the highly pathogenic Lassa virus in Africa, as well as Junin, Machupo, Guanarito, and Sabiá viruses in South America (http://www3.niaid.nih.gov/Biodefense/Research/biotresearchagenda.pdf).
- *Rift Valley Fever*. NIAID is also supporting efforts to develop vaccines against Rift Valley fever virus, a Category A pathogen of both human and veterinary importance. Vaccine candidates are being developed using a variety of approaches, including live-attenuated viruses, virus-like particles, and Sindbis virus replicon-particle-based vaccine.
- The *Food and Waterborne Diseases Integrated Research Network (FWD IRN)*. FWD IRN is supporting multidisciplinary research and the development of products to rapidly identify, prevent, and treat food- and water-borne diseases. The network currently funds:
  - Research and development of improved diagnostics for enteric pathogens.
  - Vaccine research on tularemia vaccine strain LVS, *Shigella*, and *S. typhi*. A clinical study to improve response to *S. typhi* vaccination.
  - Research on the molecular evolution and transmission of antibiotic-resistant genes in enteric pathogens.
  - Development of animal models for botulinum neurotoxins, Shiga toxin–producing *E. coli* (STEC)-mediated hemolytic uremic syndrome (HUS), *Campylobacter*-mediated enteritis, shigellosis, and Crohn's disease.
  - Strain repository for STEC.
  - Development of high-throughput screening assays for small-molecule drugs against BoNT and Shiga toxin.
  - Basic and applied research on *Clostridium difficile*, a pathogen emerging with increased virulence.
- *Botulism*. NIAID is funding research focused on the discovery and development of botulism therapeutics that would be effective in a postexposure scenario; there are two categories of potential postexposure treatments.

  NIAID is also funding research to develop protective vaccines against botulism serotypes C, D, E, F, and G and to successfully formulate vaccines that protect against multiple serotypes, including serotypes A and B. Approaches that are being funded include (i) the traditional approach of a vaccine derived from recombinant protein fragments of the neurotoxin; and (ii) novel approaches such as constructing Alphavirus replicon particles that express protective, nontoxic fragments of the neurotoxin or immune enhancing entities *in vivo*. NIAID has also used the simplified acquisition process authority provided by *Project BioShield* to contract for the further development of a serotype E–specific vaccine candidate.
- *Neurotoxin Research*. With regard to inhibitors that prevent the neurotoxins from entering neuronal cells (their site of action), NIAID is funding the discovery and development of human-compatible monoclonal antibody inhibitors. NIAID is also supporting work on human-compatible polyclonal antibodies produced in transgenic animals. NIAID has also used the simplified acquisition process authority provided by *Project BioShield* to contract for the further development of serotype A–specific monoclonal antibodies.

  With regard to inhibitors that block the activity of neurotoxin after they have entered the neuronal cell (which would provide the greatest therapeutic value), NIAID is funding, through grants and the FWD IRN contract, research on identifying inhibitors of protease activity, and the development of novel drug carrier systems to

deliver inhibitors to the interior of peripheral cholinergic nerve cells.

- *Tularemia*. NIAID is extensively supporting basic research and product development in tularemia. Thus, a Phase I clinical trial is under way using the Army's Live Vaccine Strain (LVS) tularemia vaccine through collaboration with the Department of Defense. NIAID also has a contract with DynPort Co. to support the current clinical trial and the manufacture of an additional clinical batch of LVS for possible future trials, including testing the vaccine's stability. NIAID-supported researchers have discovered critical host-defense mechanisms against tularemia in a mouse model. In research jointly supported with the Department of Defense, a real-time PCR test to simultaneously detect the bacteria that cause tularemia, plague, anthrax, and *Burkholderia* has been developed. In 2006, NIAID awarded two contracts (University of New Mexico and Dynport Co.) for the development of new tularemia vaccine candidates and the assays and animal models with which to evaluate them.
- *Plague*. NIAID is supporting a robust portfolio of basic research and product development for basic research and product development for plague. In 2006, NIAID exercised an option on its contract with Avecia, Inc., for the further development, testing, and evaluation of a plague vaccine, to include additional manufacture of GMP material for Phase II clinical trials. Together with USAMRIID and FDA, NIAID is evaluating licensed antibiotic therapies in a monkey model of pneumonic plague. Studies on gentamicin and ciprofloxacin have been completed and task orders recently given for studies on levofloxacin and doxycycline.
- *Category B and C Agents*

  - *Severe Acute Respiratory Syndrome (SARS) Coronavirus*. The Vaccine Research Center contracted with Vical, Inc., to manufacture a single, closed, circular DNA plasmid–based vaccine encoding the S protein of the SARS coronavirus. Mouse studies conducted at VRC have demonstrated that this vaccine induces T-cell and neutralizing antibody responses, as well as protective immunity. A Phase I open-label clinical study to evaluate safety, tolerability, and immune response was completed in 2006 *(387)*. In the study, healthy 18- to 50-year-old subjects received 4.0-mg DNA vaccinations at three 1-month intervals. Interim study results indicated that the vaccine is well tolerated; immunogenicity analysis of the stored samples is ongoing.
  - *West Nile Virus*. The VRC is currently developing a DNA-based vaccine against West Nile virus (WNV) in collaboration with Vical, Inc. The vaccine is based on an existing codon-modified gene-based DNA plasmid vaccine platform designed to express WNV proteins. In 2005, following preclinical safety studies and viral challenge studies, the Vaccine Research Center initiated a Phase I clinical trial to evaluate safety, tolerability, and immune responses of this recombinant DNA vaccine in human volunteers. Also in collaboration with Vical, Inc., the VRC has developed a second-generation DNA vaccine using an improved expression vector expressing the same WNV proteins.
  - *Japanese Encephalitis Virus (JEV)*. NIAID is supporting the development of a novel inactivated vaccine against Japanese encephalitis virus, using promising vaccine and adjuvant technologies. The Japanese encephalitis virus is the leading cause of encephalitis worldwide, and new vaccines against this disease are sorely needed.
  - *Influenza Therapeutics*. In 2006, NIAID made grant awards using authorities granted by the *Project BioShield Act* of 2004 for the development of high-throughput assays to screen influenza therapeutics.

- *Immunity and Biodefense*

  - *Immune Epitope Discovery*. Several of the Immune Epitope Discovery contracts made significant progress in 2004–2005 in identifying antibody and T-cell epitopes to such pathogens as influenza, vaccinia virus, and *Clostridium botulinum* neurotoxins. Investigators at Scripps Research Institute identified three candidate neutralizing antibody Fab fragments with specificity toward *Clostridium botulinum* neurotoxin A. Further characterization of these antibodies and the epitopes recognized is ongoing. Researchers at the Benaroya Research Institute at Virginia Mason characterized 18 MHC class II epitopes recognized by human $CD4^{+}$ T-cells. Finally, investigators at the La Jolla Institute of Allergy and Immunology identified 49 MHC class I epitopes recognized by $CD8^{+}$ T-cells from individuals receiving Dryvax vaccine; nine of these epitopes were recognized by T-cells from several donors and may represent common epitopes for monitoring host responses to vaccination or for developing subunit vaccines.
  - *Atopic Dermatitis and Vaccinia Immunization Network (ADVN)*. In 2004, NIAID established the Atopic Dermatitis and Vaccinia Immunization Network (ADVN) to develop short- and long-term approaches to reduce the incidence and severity of eczema vaccinatum and protect individuals with atopic dermatitis from the adverse consequences of vaccinia exposure. The ADVN consists of (i) Clinical Studies Consortium; (ii) Animal Studies Consortium; and (iii) Statistical and Data Coordinating Center.

- *Systems Approach to Innate Immunity and Inflammation.* The NIAID-supported cooperative agreement Systems Approach to Innate Immunity and Inflammation, which uses systems biology approaches to produce a detailed map of innate immune responses to infection, generated an additional 25 monoclonal antibodies to human and mouse innate immune response genes, thus bringing the total to more than 60. Many of these antibodies are being submitted to the NIAID's Biodefense Research Resource Repository for public distribution. The researchers also produced a total of 69 mutant mouse lines, using random mutagenesis techniques, with defects in immune response genes. Thirty-two of these mutations affect the immune system, 27 of the genes were identified, and 13 were shown to be involved in Toll-like receptors (TLR) signaling pathways of the innate immune responses to viral and bacterial infections. Many of the mutant mice have been deposited in existing mouse repositories (i.e., Jax, NCRR Mutant Mouse Regional Resource Centers) for public distribution.

- *Research Resources*
NAIAD has made available a number of research resources (http://www3.niaid.nih.gov/topics/BiodefenseRelated/Biodefense/research/resources/default.htm) available as follows:
  - *National Biocontainment Laboratories and Regional Biocontainment Laboratories*. NIAID is continuing to support its network of National Biocontainment Laboratories (NBLs) and Regional Biocontainment Laboratories (RBLs) by providing funds for the maintenance and operation of the NBLs.
  - *The C. W. Bill Young Center for Biodefense and Emerging Infectious Diseases* opened on the NIH campus in Bethesda, Maryland, in May 2006. The center contains BSL-2/3 laboratory space *(387)*. In addition, NIAID is constructing two other intramural BSL-3/4 laboratories, one in collaboration with USAMRIID in Frederick, Maryland, and the other at the Rocky Mountain Laboratory in Hamilton, Montana, as well as funding the upgrade to BSL-3+ of one of its *in vivo* screening contract laboratories at Utah State University to allow the study of pathogenic strains of influenza in a mouse model.
  - *NIAID Vaccine Immune T-Cell and Antibody Laboratory*. The VRC completed the development of the NIAID Vaccine Immune T-Cell and Antibody Laboratory (NVITAL) in collaboration with DAIDS and the Henry Jackson Foundation to create added immune assay capacity and to accelerate the immunologic testing of candidate vaccines, including those for Category A agents such as Ebola, Lassa, and Marburg viruses. Located in Gaithersburg, Maryland, the facility provides state-of-the-art immunogenicity testing to support NIAID vaccine trials that are multinational in scope and that generate thousands of samples. High-throughput immunology assay capabilities are developed and implemented by conforming to the highest scientific standards and goals, while complying with all appropriate quality guidelines. At the start of 2006, NVITAL began its initial experiments and began sample processing. In May 2006, NVITAL completed assay validation and began clinical trials endpoint testing.
  - *The Immune Epitope Database and Analysis Resource (IEDB)* became publicly available in February 2005 (www.immuneepitope.org). The IEDB contains extensively curated antibody and T-cell epitope information from the published literature, as well as tools to predict antibody and T-cell epitopes or visualization/mapping of epitopes onto known protein structures. There are currently 17,868 unique epitopes within the database, including all published influenza antibody and T-cell epitopes and approximately 90% of the published epitopes for NIAID Category A, B, and C Priority Pathogens. This program is supported by a DAIT contract to the La Jolla Institute of Allergy and Immunology.
  - *In Vitro and Animal Models for Emerging Infectious Diseases and Biodefense Program*. This program provides a wide range of resources for *in vitro* and *in vivo* nonclinical testing of new therapies and vaccines. These contracts provide resources for development and validation of small laboratory animal and non-human primate infection models for licensure of vaccines and therapeutics by the Food and Drug Administration (FDA). Specific projects include the *in vitro* assays and animal models for anthrax, plague, tularemia, smallpox, ricin, botulinum neurotoxin, SARS, and avian influenza, as well as other antimicrobials.

More information on the numerous NIAID resources available to biodefense researchers is provided at http://www2.niaid.nih.gov/biodefense/research/resources.htm.

23.16.2 Genomics and Proteomics

NIAID has made a significant investment in the genomic sequencing of microorganisms considered agents of bioterrorism. Knowledge of the genomes of these organisms will aid researchers in discovering new targets for therapeutics, vaccines, and diagnostics. To this end, NIAID is currently supporting sequencing efforts of multiple NIAID Category A, B, and C potential agents of bioterrorism. As of September 2006, NIAID-supported investigators completed

131 genome sequencing projects for 105 bacteria, 8 fungi, 15 parasitic protozoans, 2 invertebrate vectors of infectious diseases, and 1 plant. In addition, NIAID completed the sequence for 1,467 influenza genomes.

In 2006, genome sequencing projects (see also Section 25.4.2.1 in Chapter 25) were completed for the following organisms:

Burkholderia mallei (3 strains)
Burkholderia pseudomallei (3 strains)
Burkholderia cenocepacia
Burkholderia dolosa
Campylobacter (9 strains)
Coxiella burnetii (2 strains)
Escherichia coli (1 strain)
Listeria monocytogenes (2 strains)
Mycobacterium tuberculosis (2 strains)
Pseudomonas aeruginosa (3 strains)
Rickettsiella grylli (1 strain)
Shigella dysenteriae
Yersinia pestis (1 strain)
Influenza viruses (1,134 additional isolates)
Aspergillus fischerianus
Aspergillus clavatus
Entamoeba invadens
Entamoeba dispans
Plasmodium falciparum (2 strains)
Toxoplasma gondii (1 strain)
Trichomonas vaginalis
Ricin communis

Ongoing Sequencing Projects. In 2006, NIAID supported approximately 40 large-scale genome sequencing projects for additional strains of viruses, bacteria, fungi, parasites, viruses and invertebrate vectors, and new projects include additional strains of *Borrelia, Clostridium, E. coli, Salmonella, Streptococcus pneumonia, Ureaplasma, Coccidioides, Penicillium marneffei, Talaromyces stipitatus, Lacazia loboi, Histoplasma capsulatum, Blastomyces dermatitidis, Cryptosporidium muris*, dengue viruses, and additional sequencing and annotation of *Aedes aegyptii*.

For more information on the NIAID's genomic program, including resources available to researchers, see Section 25.4.2 in Chapter 25.

23.16.3 Recent NIAID Research Initiatives

Numerous NIAID initiatives resulted in 2006 biodefense awards in the areas of basic research, product development, clinical research, and research resources. For a comprehensive list of awards, see the 2006 Biodefense Awards page at http://www3.niaid.nih.gov/Biodefense/Research/2006awards/default.htm.

- *Basic Research*
  - *Biodefense and Emerging Infectious Diseases Research Opportunities*. Objective: To encourage the submission of investigator-initiated research grant applications in biodefense and select emerging infectious diseases. The goal is to expedite research leading to the diagnosis, prevention, and treatment of diseases caused by potential bioterrorism agents.
  - *Innate Immunity to NIAID Category B Protozoa*. Objective: To discover the cellular/molecular/ biochemical mechanisms by which the mammalian innate immune system responds to NIAID Category B Priority Pathogens.
  - *Training and Career Development for Biodefense and Emerging Diseases*. Objective: To ensure that an adequate cadre of well-trained and motivated investigators are available to pursue research and development objectives in biodefense and emerging diseases.
- *Product Development*
  - *Assays for Influenza Therapeutics: Project BioShield*. Objective: To develop high-throughput *in vitro* screening assays for influenza antiviral therapeutics incorporating validated, high-priority biochemical targets.
  - *Development of Therapeutic Agents for Selected Viral Diseases*. Objective: To develop new, safe, and effective therapeutics for variola major and viral hemorrhagic fevers, viral encephalitis, and influenza.
  - *NIAID Small Business Biodefense Program*. Objective: To encourage SBIR/STTR applications to develop therapeutics, vaccines, adjuvants/immunostimulants, diagnostics, and selected resources for biodefense.
  - *Cooperative Research Partnerships for Biodefense*. Objective: To support the discovery, design, and development of vaccines, therapeutics, adjuvants, and diagnostics for NIAID Category A, B, and C priority pathogens and toxins.
- *Research Resources*
  - *NBL Operations Cooperative Agreements*. Objective: To support operations of NIAID National Biocontainment Laboratories (NBLs).
  - *Services for Pre-Clinical Development of Therapeutic Agents*. Objective: To establish a resource to facilitate preclinical development of therapeutic agents (drugs or biological products) including activities required for Investigational New Drug applications.

23.17 Recent Scientific Advances

- *An Orally Bioavailable Antipoxvirus Compound (ST-246) Inhibits Extracellular Virus Formation and Protects Mice from Lethal Orthopoxvirus Challenge*. Concern over the use of smallpox (variola virus) as a biological weapon has prompted interest in the development of small-molecule therapeutics against the disease. Smallpox is highly transmissible and causes severe disease with high mortality rates. ST-246 has been shown to be highly active against a number of orthopoxviruses both *in vitro* and in animal models. Cowpox virus variants selected in cell culture for resistance to ST-246 were found to have a single amino acid change in the V061 gene. Re-engineering this change back into the wild-type cowpox virus genome conferred resistance to ST-246, suggesting that V061 is the target of ST-246 antiviral activity. The cowpox virus V061 gene is homologous to vaccinia virus F13L gene, which encodes a major envelope protein (p37) required for production of extracellular virus. In cell culture, ST-246 inhibited plaque formation and virus-induced cytopathic effects. In single-cycle growth assays, ST-246 reduced extracellular virus formation 10-fold relative to untreated controls, while having little effect on the production of intracellular virus. *In vivo*, oral administration of ST-246 protected BALB/c mice from lethal injection, after intranasal administration of $10 \times 50\%$ lethal dose (LD_{50}) of vaccinia virus strain IHD-J. ST-246-treated mice that survived infection acquired protective immunity and were resistant to subsequent challenge with lethal dose ($10 \times LD_{50}$) of vaccinia virus. Furthermore, orally administered ST-246 also protected A/NCr mice from lethal infection, after intranasal inoculation with $40,000 \times LD_{50}$ of ectromelia virus. These and other results, coupled with its lack of toxicity, make ST-246 a superb candidate for further development and eventual licensure by the FDA to prevent or treat smallpox infection in humans *(524)*.
- *Activity and Mechanism of Action of N-Methanocarbathymidine Against Herpesvirus and Orthopoxvirus Infections*. *N*-Methanocarbathymidine [(*N*)-MCT] is a new conformationally locked analogue that was found to exert *in vitro* activity active against some herpesviruses and orthopoxviruses *(525)*. The antiviral activity of (*N*)-MCT was dependent on the type I thymidine kinase (TK) in herpes simplex virus and also appeared to be dependent on the type II TK expressed by cowpox and vaccinia viruses, suggesting that it is a substrate for both of these divergent forms of the enzyme. Furthermore, (*N*)-MCT was also found to exhibit good activity inhibiting viral DNA polymerase once it is activated by the viral TK homologs. This mechanism of action has explained the rather unusual spectrum of activity, which was limited to only orthopoxviruses, alphaherpesviruses, and the Epstein-Barr virus, as all of these viruses express molecules with TK activity that can phosphorylate and thus activate (*N*)-MCT.
- *Conserved Receptor-Binding Domains of Lake Victoria Marburg Virus and Zaire Ebola Virus Bind a Common Receptor*. The $GP_{1,2}$ envelope glycoproteins (GPs) of the filoviruses (Marburg and Ebola viruses) mediate cell-surface attachment, membrane fusion, and entry into permissive cells. It has been reported that a 151-amino-acid fragment of the Lake Victoria Marburg virus GP_1 subunit bound Filovirus-permissive cell lines more efficiently than did a full-length GP_1 *(526)*. Furthermore, a homologous 148-amino-acid fragment of the Zaire Ebola virus GP_1 subunit similarly bound the same cell lines more efficiently than did a series of longer GP_1 truncation variants. Neither the Marburg virus GP_1 fragment nor that of Ebola virus bound a nonpermissive lymphocyte cell line. Both fragments specifically inhibited the replication of infectious Zaire Ebola virus, as well as the entry of retroviruses pseudotyped with either Lake Victoria Marburg virus or Zaire Ebola virus $GP_{1,2}$. These studies have identified the receptor-binding domains of both viruses, indicating that these viruses use a common receptor, and suggested that a single small molecule or vaccine can be developed to inhibit infection by all filoviruses *(526)*.
- *SV2 is the Protein Receptor for Botulinum Neurotoxin A*. The botulinum toxins (BoNTs) rank among the most toxic substances known and are responsible for food poisoning cases with high morbidity and mortality. These toxins are also potential weapons for bioterrorism, and are currently being used to treat a variety of medical conditions. How the widely used BoNT/A recognizes and enters neurons is poorly understood. A group of researchers at the University of Wisconsin found that BoNT/A enters neurons by binding to the synaptic vesicle protein SV2 (isoforms A, B, and C) *(527)*. Synaptic vesicles served the role of transporting neural cell signaling molecules (transmitters) from the neural cell to the muscle cell to mediate movement. When these vesicles reach the junction between neural cells and muscle cells to release the transmitters, the toxin, which has been absorbed to the bloodstream, takes advantage of the temporary exposure of SV2 to the circulation by binding to SV2. As result, the toxin enters into the neural cell when the vesicle returns to the interior of the cell. Once within the neural cell, the toxin will act as an enzyme to disrupt further transport of transmitters by the vesicles. By dissecting the mechanism by which these toxins gain entry into target cells, new strategies for prophylaxes and therapeutics can be developed. This new information may enable more effective use of the toxins for treatment of conditions such as dystonia, strabismus, and migraine headache.

- *Identification and Characterization of Potent Small-Molecule Inhibitor of Hemorrhagic Fever New World Arenaviruses*. Currently, there are no virus-specific treatments approved for use against Arenavirus hemorrhagic fevers. Ribavirin is the only compound that has shown partial efficacy against some Arenavirus infections, but with a high level of undesirable secondary reactions. ST-294 was discovered via *in vitro* screening and was optimized through iterative chemistry, resulting in a specific small-molecule inhibitor with selective activity against human pathogenic New World arenaviruses (Junín, Machupo, Guanarito, and Sabiá) *(528)*. ST-294 demonstrated favorable pharmacodynamic properties that permitted the demonstration of *in vivo* anti-Arenavirus activity in a newborn mouse model. ST-294 and its related compounds represent a new class of inhibitors that may warrant further development for potential inclusion in a strategic stockpile.
- *Development of High-Throughput Assays for Drug Discovery Against West Nile Virus (WNV)*. Although genetic systems have been developed for many flaviviruses, their use in antiviral high-throughput screening (HTS) assays has not been well explored. In this regard, three cell-based assays for WNV have been compared, namely (i) an assay that uses a cell line harboring a persistently replicating subgenomic replicon (containing a deletion of viral structural genes); (ii) an assay that uses packaged virus-like particles containing replicon RNA; and (iii) an assay that uses a full-length reporting virus *(529)*. A *Renilla* luciferase gene was engineered into the replicon or into the full-length viral genome to monitor viral replication. Potential inhibitors could be identified through suppression of luciferase signals upon compound incubation *(530–532)*.
- *Genomic Sequence and Analysis of a Vaccinia Virus Isolate.* In a recent study, the genomic sequence of a vaccinia virus isolate (VACV-DUKE) from a patient diagnosed with progressive vaccinia was determined, and its availability for the first time allowed a genomic sequence of vaccinia virus isolate associated with a smallpox vaccine complication (necrosum) to be analyzed and compared with the genomic sequence of culture-derived clonal isolates of the Dryvax vaccine *(533)*. The study showed that both sequences were overall very similar and that virus in lesions that resulted from progressive vaccinia after vaccination with Dryvax are likely clonal in origin. Although other clones derived from Dryvax vaccine have been sequenced, VACV-Duke was unique in being the only completely sequenced Dryvax isolate obtained from a human source. Detailed analysis of its nucleotide sequence and gene content will allow for better understanding of the population diversity of Dryvax, and the lack of sequence heterogeneity is suggestive of a single clonal lesion source.
- *A Novel Cell Culture–Derived Smallpox Vaccine in Vaccinia-Naïve Adults.* In the context of the potential use of smallpox as a biological weapon, the development of a new generation of smallpox vaccines represents an important part of a viable and efficient biodefense strategy. A Phase II randomized, double-blind, controlled trial was conducted to determine whether a clonal smallpox vaccine, ACAM2000, manufactured in cell culture, was equivalent to the standard calf-lymph vaccine, Dryvax, in terms of cutaneous response rate, antibody responses, and safety *(534)*. All subjects in the highest ACAM2000 dose group and the Dryvax group experienced a successful vaccination. Dilution doses of ACAM2000 were associated with success rates below the 90% threshold established for efficacy. There were no differences in the proportion of subjects who developed neutralizing antibody: 94% in the highest ACAM2000 dose group (95% CI, 84 to 99) and 96% in the Dryvax group (95% CI, 86 to 100). In addition, no significant differences were seen between the effective ACAM2000 and Dryvax groups regarding the occurrence of adverse effects.
- *Safety and Immunogenicity of IMVAMUNE as a Third Generation of Smallpox Vaccine*. A Phase I trial was performed to investigate the safety and immunogenicity of a third-generation of smallpox vaccine MVA-BN (IMVAMUNE), a highly attenuated clone derived from the modified vaccinia Ankara (MVA) virus strain 571, in naïve and pre-immunized subjects *(535)*. The vaccine was administered to healthy subjects in five different doses and routes of administration. All vaccinations were well tolerated, with the most frequent symptom being mild to moderate pain at the injection site. The IMVAMUNE vaccine has the potential to be developed as an efficient and safe alternative to the conventional smallpox vaccine such as Lister-Elstree or Dryvax. Unique attributes render it a promising candidate for prophylactic mass immunization even in subjects for whom conventional smallpox vaccines are contraindicated.
- *Modified Vaccinia Ankara Virus Protects Macaques Against Respiratory Challenge with Monkeypox Virus*. The use of classic smallpox vaccines based on vaccinia virus has been associated with severe complications in both naïve and immune individuals. The modified vaccinia Ankara (MVA) vaccine, a highly attenuated replication-deficient strain of vaccinia virus, has been proven to be safe in humans and immunocompromised animals. In a recent study, the efficacies of MVA alone and in combination with classic vaccinia virus–based vaccines were compared in a cynomolgus macaque monkeypox model *(536)*. The MVA-based smallpox vaccine protected macaques against lethal respiratory challenge

with monkeypox virus and should therefore be considered an important candidate for protecting humans against smallpox.

- *Effective Antimicrobial Regimens for Use in Humans for Therapy of Bacillus anthracis Infections and Post-Exposure Prophylaxis.* The objective of this study was to identify a levofloxacin treatment regimen that would serve as an effective therapy for *Bacillus anthracis* infections and as a postexposure prophylaxis *(537)*. An *in vitro* hollow-fiber infection model that replicates the pharmacokinetic profile of levofloxacin observed in humans [half-life ($t_{1/2}$), 7.5 hours] or in animals, such as the mouse or the rhesus monkey ($t_{1/2}$, ∼2 hours), was used to evaluate a proposed indication for levofloxacin (500 mg, once daily) for treating *Bacillus anthracis* infections. The results obtained with the *in vitro* model served as the basis for the doses and the dose schedules that were evaluated in the mouse inhalational anthrax model. The effects of levofloxacin and ciprofloxacin treatment were compared with those of no treatment (untreated controls). The main outcome measure in the *in vitro* hollow-fiber infection model was a persistent reduction of culture density ($\geq$ 4 $\log_{10}$ reduction) and prevention of the emergence of levofloxacin-resistant organisms. In the mouse inhalational anthrax model, the main outcome measure was survival. The results indicated that levofloxacin given once daily with simulated human pharmacokinetics effectively sterilized *Bacillus anthracis* cultures. By using a simulated animal pharmacokinetic profile, a once-daily dosing regimen that provided a human-equivalent exposure failed to sterilize the cultures. Dosing regimens that "partially humanized" levofloxacin exposures within the constraints of animal pharmacokinetics reproduced the antimicrobial efficacy seen with human pharmacokinetics. In a mouse inhalational anthrax model, once-daily dosing was significantly inferior (survival end point) to regimens of dosing every 12 hours or every 6 hours with identical total daily levofloxacin doses. These results demonstrate the predictive value of the *in vitro* hollow-fiber infection model with respect to the success or the failure of treatment regimens in animals. Furthermore, the model permits the evaluation of treatment regimens that "humanize" antibiotic exposures in animal models, enhancing the confidence with which animal models may be used to reliably predict the efficacies of proposed antibiotic treatments in humans in situations (e.g., the release of pathogens as agents of bioterrorism or emerging infectious diseases) where human trials cannot be performed. A treatment regimen effective in rhesus monkeys was identified *(537)*. This study demonstrated the combinational use of *in vitro* hollow-fiber and animal models to evaluate the effectiveness of certain antibiotics for treating human infections for diseases where human trials cannot be performed, such as anthrax and plague. Such systemic pharmacokinetic and pharmacodynamic characterization of existing antibiotics will allow scientists to identify agents and to design effective treatment regimens. The findings gained from this study will provide the public with options of more than one antibiotic if there is an urgent need to counteract a bioterror attack or other unexpected outbreak of an emerging infectious disease.
- *Mutual Enhancement of Virulence by Enterotoxigenic and Enteropathogenic Escherichia coli*. Enterotoxigenic and enteropathogenic *Escherichia coli* (ETEC and EPEC, respectively) are common causes of diarrhea in children, especially in developing countries. Dual infections by both pathogens have been noted fairly frequently. It has been previously shown that cholera toxin and forskolin markedly potentiated EPEC-induced ATP (adenosine 5′-triphosphate) release from the host cells, and this potentiated release was found to be mediated by the cystic fibrosis transmembrane conductance regulator. A follow-up study *(538)* examined whether the ETEC heat-labile toxin (LT) or the heat-stable toxin (STa, also known as ST) potentiated the EPEC-induced ATP release. The results showed that crude ETEC culture filtrates, as well as purified ETEC toxins, did potentiate EPEC-induced ATP released in cultured T84 cells. Co-infection of T84 cells with live ETEC plus EPEC bacteria also resulted in enhanced ATP release compared with that of EPEC alone. These and other studies have demonstrated that ETEC toxins and EPEC-induced damage to the host cells both enhanced the virulence of the other type of *E. coli (538)*.
- *Peptidoglycan Recognition Proteins as a New Class of Human Bactericidal Proteins*. Skin and mucous membranes come into contact with the external environment and protect tissues from infections. As one of the components of the innate immune system, the skin and mucous membranes produce antimicrobial peptides, such as defensins. These antimicrobial peptides damage the bacterial cell membrane, thereby protecting the host from infection. The existence of a novel class of human bactericidal and bacteriostatic proteins that are present in the skin, eyes, salivary glands, throat, tongue, esophagus, stomach, and intestine has been recently discovered *(539)*. Thus, the peptidoglycan recognition proteins 3 and 4 (PGLYRP3 and PGLYRP4) are secreted as 89- to 115-kDa disulfide-linked homo- and heterodimers and are bactericidal against several pathogenic and nonpathogenic transient but not normal flora, Gram-positive bacteria. Furthermore, PGLYRP3 and PGLYRP4 were bacteriostatic toward all other tested bacteria, which included Gram-negative bacteria. PGLYRP3 and PGLYRP4 have shown a mechanism of action distinct from their antimicrobial peptide counterparts, as well as

different structures and expression patterns. These novel proteins interact with the bacterial cell wall peptidoglycan as a means of killing. *Listeria monocytogenes* was highly sensitive to killing by both PGLYRP3 and PGLYRP4. Furthermore, both were active *in vivo*, protecting mice in a *S. aureus* lung infection model. This novel discovery could ultimately have implications for the development of broad-spectrum vaccines and immunotherapeutics.

- *The Unc93b1 Mutation Is Affecting Both Innate and Adaptive Immune Responses*. By using *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis in mice, researchers have identified a recessive mutant that is a single-base transversion of an allele of UNC-93B (3d) *(540)*. In addition, by positional identification it was found that the 3d mutant was a missense allele of *Unc93b1*, which encodes the 12-membrane-spanning protein UNC-93B, a highly conserved molecule found in the plasma reticulum with multiple paralogs in mammals. The 3d mutant is defective in its response to foreign nucleic acids and cannot signal through the Toll-like receptors (TLR) 3, 7, and 9 of the innate immune system. Using mice with the 3d mutation, an additional function of this protein was discovered that affects the adaptive immune response, independent of TLR signaling. The 3d mutation led to defective presentation of exogenous antigen, abolished cross-priming for $CD8^+$ T-cells, and inhibited $CD4^+$ T-cell priming. Innate responses to nucleic acids and exogenous antigen presentation, both of which begin in the endosomes, seemed to depend on an endoplasmic reticulum–resident protein, which is suggestive of communication between these organellar systems. Hence, the protein has two seemingly unrelated functions, which affect both the innate immune system and the adaptive immune response *(540)*.
- *Systems Biology Revealed a Novel Function of the Transcription Factor ATF3*. Although the innate immune system is absolutely required for host defenses, when uncontrolled it leads to inflammatory disease. This control is mediated, in part, by cytokines that are secreted by macrophages. Because the immune regulation is extraordinarily complex, it can be best investigated with systems approaches—that is, using computational tools to predict regulatory networks arising from global, high-throughput data sets. Using cluster analysis of a comprehensive set of transcriptomic data derived from Toll-like receptor (TLR)-activated macrophages, researchers have identified a prominent group of genes that appeared to be regulated by activating transcription factor 3 (ATF3), a member of the CREB/ATF family of transcription factors *(541)*. Network analysis predicted that ATF3 would be part of a transcriptional complex that also contained members of the nuclear factor NF-κB family of transcription factors. Furthermore, promoter analysis of the putative ATF3-regulated gene cluster demonstrated an overrepresentation of closely apposed ATF3 and NF-κB binding sites, which was verified by chromatin immunoprecipitation and hybridization to a DNA microarray. In other studies, microarray analysis was used to identify gene transcription factors affected in macrophages at different time points after LPS activation through Toll-like receptor 4. Predictions resulting from the analysis were validated in a number of biochemical assays using LPS-activated macrophages. For example, at early time points, activating transcription factor 3 (ATF3) was found to be a negative regulator of LPS-induced gene expression for IL-6 and IL-12 cytokine production. An *in vivo* model confirmed these findings, as mice deficient in ATF3 were much more susceptible to septic shock. A potential mechanism was described that involves ATF3 binding to histone deacetylase (HDAC) to alter chromatin structure, resulting in inhibition of IL-6 and IL-12 gene transcription. In addition, several useful software tools were developed by this group to predict which regulatory circuits operate under particular activation conditions.
- *Cytokine Milieu of Atopic Dermatitis Skin Subverts the Innate Immune Response to Vaccinia Virus*. Atopic dermatitis (AD) is associated with eczema vaccinatum (EV), a disseminated viral skin infection that follows inoculation with vaccinia virus (VV). A recent study *(542)* examined whether atopic dermatitis skin can control the replication of vaccinia virus, as well as the role of IL-4 and IL-13 in modulating the human cathelicidin LL-37, an antimicrobial peptide that kills vaccinia virus. The results showed that AD skin exhibited increased VV replication and decreased LL-37 expression compared with that of normal or psoriatic skin. Furthermore, IL4/IL-13 enhanced VV replication while downregulating LL-37 in VV-stimulated keratinocytes. Neutralizing IL-4/IL-13 in AD skin augmented LL-37 and inhibited VV replication. Cathelicidins were induced via Toll-like receptor 3 and were inhibited by IL-4/IL-13 through STAT-6. Skin from cathelicidin-deficient mice exhibited reduced ability to control VV replication. Exogenous LL-37 controlled replication of vaccinia virus in infected keratinocytes and atopic dermatitis skin explants. The overall results from the study demonstrated that Th2 cytokines enhanced VV replication in AD skin by subverting the innate immune response against vaccinia virus in a STAT-6-dependent manner *(542)*.
- *Immune Protection of Non-Human Primates Against Ebola Virus with Single Low-Dose Adenovirus Vectors Encoding Modified Glycoproteins*. Ebola virus causes a hemorrhagic fever syndrome that is associated with high mortality in humans. In the absence of effective therapies for Ebola virus infection, the development of a vaccine becomes an important strategy to contain outbreaks. Immunization with DNA and/or replication-defective

adenoviral vectors (rAd) encoding the Ebola glycoprotein (GP) and nucleoprotein (NP) has been previously shown to confer specific protective immunity in non-human primates. Furthermore, GP can exert cytopathic effects on transfected cells *in vitro*, and multiple GP forms have been identified in nature, raising the question of which would be optimal for a human vaccine. To address this question, VRC-led researchers have explored the efficacy of mutant GPs from multiple Ebola virus strains with reduced *in vitro* cytopathicity and analyzed their protective effects in the primate challenge model, with or without NP *(543)*. Deletion of the GP transmembrane domain eliminated *in vitro* cytopathicity but reduced its protective efficacy by at least one order of magnitude. In contrast, a point mutation was identified that abolished this cytopathicity but retained immunogenicity and conferred immune protection in the absence of NP. The minimal effective rAd dose was established at 10^{10} particles, two logs lower than that used previously. Expression of specific GPs alone vectored by rAd were found sufficient to confer protection against lethal challenge in a relevant non-human primate model. Elimination of NP from the vaccine and dose reductions to 10^{10} rAd particles did not diminish protection and simplify the vaccine, providing the basis for selecting a human vaccine candidate *(543)*.

- *Protection Against Multiple Influenza A Subtypes by Vaccination with Highly Conserved Nucleoprotein*. Current influenza vaccines elicit antibodies effective against specific strains of the virus, but new strategies are urgently needed for protection against unexpected strains. DNA vaccines have been shown to provide protection in animals against diverse virus strains, but the potency of the vaccines needs improvement. Scientists at the Vaccine Research Center tested a DNA prime-recombinant adenoviral boost vaccine targeted at one of the influenza viral proteins, nucleoprotein (NP). Strong antibody and T-cell responses were induced. Protection against viral challenge was substantially more potent than was DNA vaccination alone. Equally importantly, vaccination protected against lethal challenge with highly pathogenic H5N1 virus. Thus, gene-based vaccination with NP may contribute to protective immunity against diverse influenza viruses through its ability to stimulate cellular immunity *(544)*.

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