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12 Biodefense and Special Pathogen Vaccines

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This chapter discusses vaccines for military and biodefense research personnel as well as some vaccines that are in development for uncommon or geographically limited diseases. Military personnel have the potential to be exposed to many infectious agents as endemic diseases and in their unnatural form as biological weapons. Increasingly, civilian populations may be targets for terrorist attacks using microorganisms (or their toxins), as was demonstrated by the purposeful dissemination of anthrax spores following ballistic attacks on the World Trade Center and the Pentagon in 2001.

The U.S. Army has had a longstanding program to develop vaccines to combat these threats. Within the U.S. Department of Defense (DOD), the U.S. Army Medical Research and Materiel Command (USAMRMC), located at Fort Detrick, Maryland, is the principal organization responsible for vaccines and other medical countermeasures against biological warfare agents. Within USAMRMC, the unit with direct responsibility for this defensive mission is the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID), also at Fort Detrick. Before 1969, when the United States maintained an offensive biological weapons program, the U.S. Army Medical Unit within the Walter Reed Army Institute of Research in Washington, DC, served in this capacity.

Many of the biowarfare vaccines currently available at USAMRIID and elsewhere to protect laboratory researchers studying these agents were conceived at the U.S. Army Medical Unit and were further developed at the now closed National Drug Laboratories (also known as The Salk Institute's Government Services Division [TSI-GSD]). Most of the vaccines developed at Fort Detrick, for a variety of reasons, remain investigational new drugs (INDs). However, all these vaccines have undergone extensive preclinical testing and have progressed to Phase II trials that have continued for many years. Meanwhile, USAMRIID researchers and others continue to apply new technology to develop improved vaccines.

Table 12.1 presents all IND vaccine products of military interest developed at USAMRMC/USAMRIID; most of these vaccines are currently available for administration only through USAMRIID's Special Immunizations Program (SIP).[1] One of the vaccines, the Candid #1 Junin (Argentine hemorrhagic fever [AHF]) vaccine, shown to be efficacious in a Phase III trial[2] in Argentina, has been incorporated into that country's public health program and administered to thousands of individuals. Several other vaccines, including Venezuelan equine encephalitis (VEE) TC-83, eastern equine encephalitis (EEE) vaccine, western equine encephalitis (WEE) vaccine, tularemia live vaccine strain (LVS), Rift Valley fever (RVF) inactivated vaccine, and the whole-cell Q fever vaccine, have also been administered to more than 1000 volunteers, primarily through the SIP. In contrast, the chikungunya (CHIK) vaccine strain 181/clone 25, live attenuated RVF vaccine MP-12, Q fever chloroform-methanol residue (CMR) vaccine, and vaccinia/Hantaan vaccine have been administered to no more than a few hundred volunteers each.

Each of the vaccines in active use in USAMRIID's SIP is administered under an approved human use protocol, and all volunteers provide written informed consent before vaccination. These IND vaccines are administered on a voluntary basis to at-risk laboratory and field workers. Most SIP participants are scientists and technicians from USAMRIID, but scientists from academia, other federal and state agencies, and private drug companies also participate.[1] Extramural scientists who wish to receive one of these vaccines must come to USAMRIID for vaccine administration and safety monitoring. The protocols and consent forms are reviewed by the USAMRIID Scientific Review Committee, the Headquarters USAMRMC Institutional Review Board, and the Office of Research Protections (within USAMRMC Headquarters at Fort Detrick) before submission to the U.S. Food and Drug Administration (FDA). Protocols are conducted in accordance with the Belmont Principles, DOD Instruction 3216.02 ("Protection of Human Subjects and Adherence to Ethical Standards in DOD-Supported Research"), and other applicable DOD and FDA regulations and guidelines. Many of these vaccines have been in use since the 1960s and 1970s, and all are subject to periodic potency and lot-release testing as required by FDA.

Continuous monitoring for safety and immunogenicity is conducted through the IND vaccine managing authority: the U.S. Army Medical Materiel Development Activity and the Non-Clinical Studies Division of USAMRIID. Funding for testing of these products is provided through the Medical Countermeasure Systems, Fort Belvoir, Virginia. To date, all vaccines have demonstrated an acceptable safety profile and reasonable immunogenicity (Table 12.2), except the botulinum toxoid pentavalent (ABCDE) vaccine, which is no longer administered.[3] The most reactogenic product is the live attenuated VEE TC-83 vaccine, which frequently induces a short-term systemic reaction.[4] The inactivated vaccines, except the whole-cell Q fever vaccine, require multiple doses for priming and frequent periodic boosting to maintain acceptable levels of neutralizing antibody. All vaccines have been administered to men and nonpregnant women within a broad range of ages, ethnicities, and races. With the exception of Junin vaccine, efficacy of these products is inferred by the absence of laboratory-acquired infection among recipients. However, because laboratory practices and engineering controls have evolved in concert with use of the vaccines, quantifying efficacy on a continuous basis is difficult.

USAMRIID has been proactive in evaluating, not only the short-term reactogenicity, but also the long-term medical safety of its vaccines and other vaccines administered to at-risk laboratory workers. One study[5] was aimed at detecting any long-term medical effects from repeated injections with multiple vaccines; a second study[6] evaluated volunteers who participated in biomedical research as part of Operation Whitecoat.

The first study consisted of 155 volunteers who had participated in a multiple immunizations program (MIP cohort) and 265 community control volunteers who had not participated in a MIP, matched by age, race, and sex.[5] The majority of study volunteers were male (83%) and older in age (average age: 69.4 years). This study did not link any disease or medical condition to repetitive immunization with multiple antigens or any single antigen. Fatigue was noted

TABLE 12.1 Investigational New Drug, Limited-Use Vaccines in Inventory at USAMRIID: Characteristics and Administration

Name	IND Number	Type	Dosage (mL)	Route	Schedule (days)	Boosters
VEE TC-83 (NDBR 102)	BB-IND 142	Live attenuated	0.5	SC	0	Boost with C84 per titer
VEE C-84 (TSI-GSD 205)	BB-IND 914	Inactivated	0.5	SC	0[a]	Yes; based on titer
WEE (TSI-GSD 210)	BB-IND 2013	Inactivated	0.5	SC	0, 7, 28	Mandatory boost at month 6[b]
EEE (TSI-GSD 104)	BB-IND 266	Inactivated	0.5	SC	0, 28	Mandatory boost at month 6[c]
Chikungunya 181/clone 25 (TSI-GSD 218)	BB-IND 2426	Live attenuated	0.5	SC	0	Not determined
RVF (TSI-GSD 200)	BB-IND 365	Inactivated	1.0	SC	0, 7, 28	Yes; based on titer[d]
RVF MP-12, ZH548 (TSI-GSD 223)[e]	BB-IND 4307	Live attenuated	1.0	IM	0	No
Junin, or Candid #1[e]	BB-IND 2257	Live attenuated	0.5	SC	0	No
Q fever (NDBR 105)[e,f]	BB-IND 610	Inactivated	0.5	SC	0	No
Q fever CMR[e] (TSI-GSD 217)	BB-IND 3516	Inactivated	0.5	SC	0	Not determined
Tularemia LVS (NDBR 101, TSI-GSD 213)	BB-IND 157	Live attenuated	0.06	Scarification	0	No

CMR, chloroform-methanol residue; EEE, eastern equine encephalitis; IM, intramuscular; IND, investigational new drug; LVS, live vaccine strain; RVF, Rift Valley fever; SC, subcutaneous; TSI-GSD, The Salk Institute's Government Services Division; USAMRIID, U.S. Army Medical Research Institute of Infectious Diseases; VEE, Venezuelan equine encephalitis; WEE, western equine encephalitis.

[a]C-84 is given only after TC-83 and titer <1:20.

[b]After month 6, boosts of the WEE vaccine are given as needed per titer.

[c]Boosts of the EEE vaccine before and after month 6 are given as needed per titer (as 0.1 mL intradermally).

[d]Initial responders to RVF (inactivated) vaccine: mandatory boost at month 6, then as needed per titer. Initial nonresponders: boost within 90 days of low titer.

[e]These vaccines are currently not in active use at USAMRIID.

[f]A skin test (using Q fever skin test antigen, MNLBR 110, a dilution of the NDBR 105) is conducted 1 week prior to vaccination with Q fever vaccine (NDBR 105).

TABLE 12.2 Assessment of Efficacy and Safety of Selected Limited-Use Vaccines

			Relative Severity and Frequency of Vaccine-Related Reactions	
Name	Tests of Effectiveness	Effective?	Systemic Severity/Frequency	Local Severity/Frequency
VEE TC-83, live	Reduction in laboratory-associated infections	Yes	+++/+++	+/+
VEE C-84, inactivated	Insufficient data	Unknown	+/+	+/+
WEE, inactivated	Reduction in laboratory-associated infections	Probably	+/++	+/+
EEE, inactivated	Reduction in laboratory-associated infections	Probably	+/++	+/++
Chikungunya, live	Insufficient data	Unknown	+/++	+/++
RVF, inactivated	Reduction in laboratory-associated infections	Yes	+/+	+/+
RVF MP-12, live	Insufficient data	Unknown	+/++	+/+
Junin, live	Formal Phase III field trial	Yes	+/+	+/+
Q fever, inactivated	Reduction in laboratory-associated infections	Probably	+/+++	+/+++
Q fever CMR, inactivated	Insufficient data	Unknown	+/++	+/+++
Tularemia LVS, live	Reduction in laboratory-associated infections	Yes	+/+++	+/+++

EEE, eastern equine encephalitis; LVS, live vaccine strain; RVF, Rift Valley fever; VEE, Venezuelan equine encephalitis; WEE, western equine encephalitis.

Severity of reactions: +, most reactions are mild, <5% are severe; ++, most reactions are mild or moderate, <15% are severe; +++, a majority of reactions are mild or moderate, ≥15% are severe.

Frequency of reactions: +, typically occur after <10% of doses; ++, typically occur after ≥10% but <30% of doses; +++, typically occur after ≥30% of doses.

Table displays an overall, qualitative assessment of the severity and frequency of reactions based on published work[2,56,83,92,311–314] as well as unpublished studies conducted at USAMRIID.

more commonly among subjects who had received multiple immunizations, but this finding was not associated with number of shots, number of antigens, or time in the MIP. Although statistically significant increases and decreases in several clinical laboratory tests were observed, none of these findings appeared to be clinically significant.

The single most important finding of this study was the greater frequency of serum monoclonal proteins observed among MIP subjects compared with control subjects. However, no associations between the presence of monoclonal proteins and specific diseases or medical conditions were seen.[5] The significance and implications of this finding are unclear, warranting further investigation. A larger study with more than 1100 volunteers who participated in the SIP has been completed and should, once the results are analyzed, elucidate these findings further. The results of this study are expected to be published in 2017.

The second study involved men entering military service in the early days of the Cold War and the Vietnam War who expressed a conscientious objection to combat. These men were offered the opportunity to serve as medical research volunteers in Fort Detrick's biological warfare defense program. More than 2000 subjects participated in what became known as Operation Whitecoat. A study to assess the current health status of Operation Whitecoat medical research volunteers who served between 1954 and 1973 was developed as a joint effort between DOD and the Seventh-Day Adventist Church.[6]

Many of the volunteers enrolled in this study had been exposed to an infectious agent, vaccine, or other biological product as part of Operation Whitecoat (study group; n = 358); others had participated in research studies as unexposed control subjects or chose not to participate at all (control group; n = 164). Volunteers completed a self-administered questionnaire regarding their health status, clinical signs and symptoms, reproductive outcomes, and diseases or conditions diagnosed by medical providers. Attempts to assess frequencies of clinical signs and symptoms or diseases by individual vaccine, individual virulent agent, and individual antibiotic/inert substance exposure yielded numbers too small for meaningful analysis for all but recipients of VEE vaccine(s) and tularemia vaccine(s) as well as individuals exposed to virulent *Coxiella burnetii*. Among these exposures, a possible association between developing asthma and receipt of tularemia vaccine(s) (but not VEE vaccine or virulent *C. burnetii*) was observed (13.3% of vaccinees vs 2.4% of control subjects). In this same cohort, an association between receipt of a tularemia vaccine and a greater frequency of chronic headaches was suggested (with 35.6% of vaccinees vs 18.3% of control subjects reporting that headaches were occasionally or more frequently a problem as opposed to never or rarely a problem); however, this association did not reach statistical significance. No adverse impact on the overall health of Operation Whitecoat volunteers could be conclusively attributed to participation in research studies at Fort Detrick.[6] Loma Linda University will continue with intermittent follow-up studies.

LIMITED-USE VACCINES AGAINST VIRAL DISEASES

Alphaviruses

The alphaviruses are a group of mosquito-borne, lipid-enveloped, positive-sense, single-stranded RNA viruses belonging to the family *Togaviridae*. Alphaviruses are responsible for two distinct clinical syndromes: fever, chills, headache, myalgias, vomiting, and encephalitis (e.g., VEE, EEE, and WEE) and fever, rash, and polyarthralgias/arthritis (e.g., CHIK, Ross River virus disease [RRVD, formerly called epidemic polyarthritis], and o'nyong-nyong). Several excellent reviews are available on the classification, epidemiology, and clinical features of these agents.[7–9] Although the DOD has developed vaccines against VEE virus (VEEV), WEE virus (WEEV), EEE virus (EEEV), and CHIK virus (CHIKV), these vaccines remain investigational. Ongoing research seeks to develop improved vaccines against these pathogens.[10] For example, a recent study using replicon technology showed that individual replicon vaccine candidates for VEE and EEE or a combined VEEV/WEEV/EEEV replicon particle vaccine elicited strong neutralizing antibodies and protection against aerosol challenge with VEEV subtype I-AB (Trinidad donkey strain) and EEEV. However, the individual WEEV replicon and the combined VEEV/WEEV/EEEV replicon vaccine elicited low levels of neutralizing antibodies to WEEV and conferred poor protection in macaques.[11] In particular, Wolfe and colleagues[12] argue that an FDA-licensed trivalent VEE/WEE/EEE vaccine—one that overcomes the safety, immunogenicity, and immune interference issues of the existing IND vaccines—is required to meet the requirements of the biodefense community. Such a vaccine also would be of great value to the public health and agricultural communities.

Experience with sequential administration of alphavirus vaccines at USAMRIID has led to a number of interesting observations relating to immunologic interference. Prior immunization with inactivated EEE and/or WEE vaccines decreases one's ability to mount a neutralizing antibody response following receipt of live attenuated VEE TC-83 vaccine. However, no interference is observed when the order of administration is reversed, such that VEE TC-83 vaccine is given first.[13] Similarly, interference occurs when two live attenuated alphavirus vaccines, VEE TC-83 and a CHIK vaccine (CHIK 181/clone 25), are administered sequentially.[14] Volunteers initially vaccinated with VEE TC-83 exhibited poor neutralizing antibody responses to live attenuated CHIK vaccine (46% response rate). Among persons initially inoculated with the CHIK vaccine or placebo who then received the live attenuated VEE TC-83 vaccine, geometric mean antibody titers to VEEV, as measured by 80% plaque reduction neutralization test ($PRNT_{80}$), were uniformly depressed in CHIK vaccine recipients compared with placebo recipients.[14] Interference may be an important consideration for the development of next-generation alphavirus vaccines, and particularly for the development of multiagent alphavirus vaccines.

Venezuelan Equine Encephalitis Virus

The two VEE vaccines available for human use as INDs (vaccines)—the live attenuated product, TC-83, and a formalin-inactivated product, C-84—both derive from the same lineage. The live attenuated TC-83 virus, a subtype I-AB strain, was isolated from a donkey brain in Trinidad and was passaged 13 times in embryonated eggs.[15] The virus was attenuated by 78 passages in fetal guinea pig heart (FGPH) cell cultures, plaque-picked in chick embryo fibroblasts (CEFs), and passaged four additional times in FGPH cell cultures.[16] The VEE TC-83 virus designation is a direct reference to the 83 passages in cell culture. VEE C-84 vaccine is formalin-inactivated and is made from the TC-83 production seed, TC-82, that has undergone one additional passage in CEFs.[17] This C-84 production seed is passaged once more in CEFs to derive the C-84 vaccine, which is inactivated with 0.1% formalin and then freeze-dried. The inactivation procedure is based on that used by Salk and colleagues[18] to inactivate the poliovirus. TC-83 and C-84 contain streptomycin and neomycin, each at a concentration

of 50 μg/mL. Laboratory infections with epizootic VEEV strains closely related to the parent strain have essentially been eliminated since the introduction of these vaccines and improvement in personal protective equipment.

At USAMRIID, immunologically naïve people at risk for exposure to VEE receive a single dose of the live attenuated TC-83 vaccine. Those who seroconvert ($PRNT_{80}$ titer ≥1:20) receive a single booster of C-84 as needed based on their titer ($PRNT_{80}$ <1:20); nonresponders to TC-83 receive a booster with C-84. The response rate to TC-83 alone is 82%.[4] When TC-83 is followed by a single boost with C-84, a combined response rate of well over 90% is observed. Female responders to TC-83 tend to have titers similar to those of male responders, but the frequency of nonresponders tends to be higher among women than men; in one study, the initial response rate among recipients of a single dose of TC-83 was 74% and 85%, respectively, for women and men. The nature of this sex difference is not understood.

Approximately 23% of persons receiving the live attenuated TC-83 sustain adverse reactions, including headache, sore throat, malaise, fatigue, myalgias, arthralgias, chills, and fever—a suite of symptoms similar to those seen following natural VEEV infection but less severe.[4] The local reaction rate is less than 5%. The inactivated vaccine C-84 has a local reaction rate of approximately 5%, but essentially no systemic reactions are associated with its administration. Diabetes mellitus, abortion, and teratogenesis have been associated—epidemiologically and/or in animal studies—with natural wild-type VEEV infection.[19–21] Before pregnancy testing prior to vaccination became available, three cases of spontaneous abortion or stillbirth were temporally related to the administration of TC-83.[22] However, VEEV was not recovered from culture of tissues in either case. (Though they were reported to FDA, these cases were never published as case reports.) Since the advent of pregnancy testing, great care has been used to ensure that women are not pregnant before administration of TC-83. Out of an abundance of caution, persons with a family history of diabetes mellitus are considered ineligible for vaccination with TC-83, despite the lack of evidence for a causal association between diabetes mellitus and VEEV infection or vaccination with TC-83.

The ideal VEE vaccine would have a high seroconversion rate (>95%) and a low reaction rate (<5%). By these standards, TC-83 is reactogenic and has a moderate response rate, as measured by neutralizing antibody. In addition, TC-83 does not protect adequately against distantly related VEEV subtype I-AB variants or the other enzootic VEEV subtypes II through VI. Finally, the manufacturing process for TC-83 requires the manipulation of infectious viral particles in a biosafety level 3 containment laboratory.[10]

A new-generation, live attenuated vaccine candidate, V3526, uses twin site-directed mutagenesis of the full-length complementary DNA clone of the virulent virus RNA.[23] V3526 has two deletion mutations—a lethal deletion at the PE-2 cleavage signal site and a suppressor mutation at site 253 of the E1 glycoprotein—that should prevent reversion to wild-type VEEV. This vaccine candidate also has limited potential for transmission by mosquitoes and elicits cross-protection against different viral strains.[10] After showing promise in preclinical studies,[24] V3526 elicited the development of impressive neutralizing antibody levels in human volunteers during Phase I clinical trials. However, the vaccine was associated with a high frequency of fever and other flu-like symptoms; thus, further development was discontinued.[25,26]

Because of the long history of frequent adverse reactions related to live attenuated VEE vaccines, the manufacturer decided to inactivate V3526 and further develop it as an inactivated vaccine to be used as a priming vaccine that would replace C-84. Testing has shown reduced infectivity by both formalin inactivation (fV3526) and gamma-irradiation (gV3526). For example, both inactivated vaccine candidates showed a loss of neurovirulence after intracerebral inoculation of suckling BALB/c mice, suggesting that the vaccines were completely inactivated. Both fV3526 and gV3526 elicited robust immune responses; furthermore, protection was demonstrated by both vaccine candidates against subcutaneous challenge with VEEV I-AB Trinidad donkey strain following two doses of either vaccine. Recently, researchers have been testing these vaccine candidates, using various adjuvants and routes of administration, against subcutaneous and aerosol challenge.[24,27,28]

In addition to fV3526 and gV3526, other technologies are being evaluated for the production of vaccines against VEE. For example, Sharma and colleagues[29] found that the hydrophobic alkylating compound 1,5-iodonaphthylazide can effectively inactivate virulent VEEV strain V3000. The resulting inactivated vaccine candidate was efficacious in protecting mice from virulent VEEV challenge, and its efficacy was enhanced by the use of adjuvants. Rossi and colleagues[30,31] developed a live attenuated vaccine using an encephalomyocarditis virus internal ribosome entry site (IRES)—a construct that inhibits translation of viral proteins in mosquito cells, thus preventing transmission by the natural VEEV vector. This IRES-based VEE vaccine fully protected mice and macaques from clinical disease after aerosol challenge with virulent VEEV.

DNA vaccines are also under development and, in protective efficacy studies, have shown promise in both mice and guinea pigs.[32] One DNA vaccine candidate has also demonstrated protection against aerosol challenge with wild-type VEEV in nonhuman primates.[33,34] A candidate based on a novel approach to DNA vaccine development, infectious DNA (iDNA), was protective in one BALB/c mouse challenge study.[35]

Western Equine Encephalitis Virus

An inactivated WEE vaccine, TSI-GSD 210, has been used at Fort Detrick since the 1970s to immunize at-risk laboratory personnel. The WEE vaccine is a lyophilized product derived from supernatant fluids of primary CEF cell cultures infected with the attenuated CM4884 strain of WEEV.[36,37] The supernatant fluid is harvested and filtered, the virus is inactivated with formalin, and the final product is lyophilized for storage at −20°C. The vaccine contains 50 μg/mL of neomycin. The primary end point used to measure immunogenicity of the WEE vaccine is the $PRNT_{80}$, with a titer of at least 1:40 considered indicative of a response.

In an analysis of data from 363 volunteers who received 0.5 mL of inactivated TSI-GSD 210 vaccine subcutaneously at days 0, 7, and 28, 151 subjects (41.6%) responded with a $PRNT_{80}$ titer of 1:40 or greater, whereas 212 subjects (58.4%) failed to achieve this neutralizing antibody titer. Of 115 initial nonresponders, 76 (66.1%) converted to responder status after a single booster. Kaplan–Meier plots showed that a regimen consisting of three initial doses and one booster induced a $PRNT_{80}$ titer of at least 1:40 lasting 1.6 years in 50% of initial responders. Local and systemic adverse events are uncommon with this vaccine. Among 363 vaccinees receiving three initial injections of the WEE vaccine, only 5 reported local or systemic reactions (P.R. Pittman, P.H. Gibbs, and T.L. Cannon, unpublished data).

No instances of occupational WEE have been documented among laboratory workers who develop neutralizing antibodies following vaccination. WEE vaccine continues to be administered as part of Phase II clinical trials; however, no efficacy trial has been conducted.

A new lot of the WEE vaccine, Western Equine Encephalitis Vaccine, Inactivated, TSI-GSD 210, Lot 3-1-92, was found to be safe and immunogenic in a recent Phase I clinical trial (Dr. Robert Rivard, USAMRIID, personal communication, 2015). In this study, WEE vaccine was administered in 0.5-mL doses subcutaneously in the upper outer aspect of the triceps in a three-dose primary series (days 0, 7, and 28) with a mandatory boost (day 180). All 10 subjects were classified as responders at day 56. For 4 of 10 subjects, the titers had waned below the acceptable level by month 6. Following the month 6 boost, all 10 subjects developed titers above 1:40, and all remained above this level for at least 1 year.

DNA-based vaccines against WEE have shown promise in challenge models in mice. One such study evaluated a DNA vaccine, pVHX-6, expressing the 26 S structural gene of WEEV strain 71 V-1658. All mice receiving four intraepidermal doses of pVHX-6 survived challenge with a homologous strain, but only 62% and 50% survived challenge with Fleming and CBA87 strains, respectively.[38] Other studies have demonstrated, in a murine model, the efficacy of DNA vaccines expressing the capsid and envelope proteins of WEEV in challenge models. In two studies, a replication-defective human adenovirus serotype-5 (HAd5) was used as a vector for vaccine delivery. In the first study, a HAd5 vector encoding E2 and E1, administered as a single dose, conferred protection against challenge with homologous and heterologous WEEV strains.[39,40] In the second study, a HAd5 vector encoding E1 alone, designated adenovirus (Ad) serotype-5-E1 (Ad5-E1), provided total protection against homologous and heterologous strains of WEEV in mice.[41] A potential drawback of this approach in humans is the widespread prevalence of antibodies against adenovirus.

Eastern Equine Encephalitis Virus

The EEE vaccine (TSI-GSD 104) is a lyophilized product originating in primary CEF cell cultures infected with the attenuated PE-6 strain of EEEV. The seed for the EEE vaccine is passaged twice in adult mice and twice in guinea pigs, then passaged nine times in embryonated eggs, followed by three passages in CEFs.[42] The supernatant fluid, which is harvested and filtered, contains 50 μg each of neomycin and streptomycin and 0.25% w/v (weight per volume) of human serum albumin, USP (U.S. Pharmacopoeia). The virus is then inactivated with 0.05% formalin. When inactivation is completed, the residual formalin is neutralized by treatment with sodium bisulfite. The final product is lyophilized for storage at −20°C.

Among 255 volunteers who received the two-dose primary series of EEE vaccine (administered subcutaneously) between 1992 and 1998, 197 (77.3%) responded with a $PRNT_{80}$ titer of 1:40 or greater. Of initial nonresponders, 66% subsequently seroconverted following receipt of an EEE vaccine booster (which is administered intradermally). Among initial responders whose titers waned over time, 98.6% responded to a booster dose of EEE vaccine. Local and systemic side effects are infrequent, occurring in less than 1% of vaccinees after the primary vaccine series and in 3.7% after the first booster. Kaplan–Meier plots show that two primary doses and one intradermal booster of TSI-GSD 104 provide satisfactory neutralizing anti-EEEV antibodies in 50% of initial responders for up to 2.2 years.[43]

Among recent findings regarding next-generation EEE vaccines, a single subcutaneous dose of an EEE vaccine candidate, attenuated via an IRES, protected 100% of vaccinated mice against intraperitoneal challenge.[44] In addition, an EEEV replicon and a combined VEEV/WEEV/EEEV replicon protected macaques from aerosol challenge.[11]

Chikungunya Virus

CHIKV can cause an acute viral syndrome in humans characterized by fever, rash, and arthritis.[45] Some people, especially those who are human leukocyte antigen (HLA)-B27–positive, may develop long-term joint involvement.[46] Although documented epidemics have occurred since the late 18th century, the virus was first isolated during the 1952–1953 Tanzanian epidemic.[47] During 2004–2006, an epidemic ravaged the Indian Ocean islands east of Madagascar—La Réunion, Mauritius–Seychelles, and Mayotte.[48] More than 2 million people have been affected since the start of the epidemic, which has since spread to the Caribbean and India, with cases also occurring in persons from the United States, Europe, and elsewhere who traveled to the Caribbean.[49–52] In December 2014, Kendrick and colleagues[53] reported on the first 11 cases of CHIK originating in the United States (Florida). Genomic sequencing of six isolates from the Indian Ocean outbreak suggests that the strain responsible for the outbreak is related to East African isolates.[49] In addition, evidence from the sequence data suggests that the CHIKV strain responsible for the Indian Ocean outbreak evolved, during the course of the outbreak, into several distinct variants. One mutation may have allowed the CHIKV to be efficiently transmitted by the most abundant mosquito species on La Réunion (*Aedes albopictus*); this may help explain the noted robustness of the epidemic.[49,52,54]

Several attempts were made, with variable success, to develop an efficacious inactivated vaccine using chick embryo cell cultures, suckling mouse brain, and African green monkey kidney.[55] A live attenuated vaccine was made from the seed of the African green monkey kidney vaccine, using CHIKV strain 15561, which had originally been isolated from an infected patient during a 1962 CHIK epidemic in Thailand.[55,56] The 11th African green monkey kidney passage of CHIKV strain 15561, made at the Walter Reed Army Institute of Research, was transferred to USAMRIID and passaged in Medical Research Council (MRC)–5 cells. After 18 passages in MRC-5 cells, CHIKV 181/clone 25 was selected as a vaccine seed strain based on biomarkers. CHIKV 181/clone 25 was efficacious in challenge models in suckling mice and in nonhuman primates.[55]

A randomized, double-blind, placebo-controlled trial of the CHIK 181/clone 25 vaccine documented a seroconversion rate of 98% among alphavirus-naïve volunteers.[56] One year after immunization, 85% of vaccinees remained seropositive. Injection site and systemic symptoms, including flu-like symptoms, were similar in vaccine and placebo recipients. However, the vaccine was temporally associated with arthralgia in 8% of vaccinees. One volunteer in the vaccine group developed a pruritic, eczema-like rash at the injection site. Interference between the CHIK 181/clone 25 vaccine and the live attenuated VEE TC-83 vaccine is discussed above. This live attenuated CHIK vaccine requires additional Phase II and Phase III clinical testing. Current epidemics in the Caribbean, Indian Ocean, and India offer unique prospects for Phase III clinical testing of this vaccine.

Additional strategies for the development of a safe and efficacious vaccine for CHIK are being pursued. These include chimeric alphavirus vaccine candidates using VEEV attenuated vaccine strain TC-83, a naturally attenuated strain of EEEV, or Sindbis virus as a backbone with the structural protein genes of CHIKV. Wang and colleagues[57] found that each of these chimeras produced robust neutralizing antibody responses, and vaccinated mice were fully protected against disease and viremia after CHIKV challenge. One DNA vaccine candidate expressing a component of the CHIKV envelope glycoprotein produced neutralizing antibodies in mice and macaques.[58] A second DNA vaccine candidate uses a plasmid coding for the CHIKV-capsid,

E1 and E2. When injected into mice, this construct induced broad cellular immunity and produced antibodies, detectable by enzyme-linked immunosorbent assay (ELISA), that recognized native antigen.[59] Another group is evaluating a formalin-inactivated Vero cell–adapted vaccine candidate prepared using a strain from the India epidemic.[60] Most recently, a promising virus-like particle (VLP) vaccine containing the viral envelope has elicited high titers of neutralizing antibodies in nonhuman primates and in human volunteers.[61–63] In addition, a single immunization with a measles virus–vectored CHIK vaccine candidate induced high CHIKV antibody titers and protected mice from a lethal CHIKV challenge.[64]

Ross River Virus

RRVD, or epidemic polyarthritis, was first recognized in Australia in 1928; Ross River virus (RRV), its causative agent, was first isolated in 1963 from a pool of mosquitoes.[65–67] RRVD is essentially limited to Australia, Fiji, and the surrounding islands, including American Samoa and the Cook Islands, where several thousand cases occur per year.[68,69] In humans, polyarthritis may be followed by fever, rash, and lethargy; symptoms generally resolve after 3 to 6 months.[69,70] In 1997, U.S. marines participating in Operation Tandem Thrust in Queensland, Australia, had an infection rate of 1.5%; RRVD developed in nine individuals.[71]

A vaccine candidate was derived from a virus isolated from a human case of classical RRVD using the C6-36 cell line (*A. albopictus*).[72] The candidate underwent four serial passages in MRC-5 human fetal lung cells followed by two passages in Vero cells. Cell cultures contained penicillin (100 U/mL) and streptomycin (100 µg/mL). The virus was inactivated using binary ethyleneimine. In mice, the vaccine induced neutralizing antibodies and conferred protection against viremia after intravenous challenge with live RRV.[73]

More recently, a Vero cell–grown, formalin-inactivated, benzonase-digested (to digest host cell DNA), sucrose gradient–purified vaccine candidate induced neutralizing antibodies in mice.[74] Immunized mice and guinea pigs failed to develop viremia following intravenous challenge with the prototype strain of RRV (T48). In a Phase I/II dose escalation study[75] in 382 healthy, RRV-naïve adults, this vaccine was safe and immunogenic when administered as three immunizations (at days 0 and 21 and month 6) at four dose levels, with or without aluminum hydroxide added as an adjuvant; the adjuvanted 2.5-µg dose stimulated the highest immune response. This group recently completed a Phase III study in 1755 healthy younger adults (ages 16–59 years) and 209 healthy older adults (ages 60 and older).[76] The vaccine was well tolerated. Neutralizing antibody titers (≥1:10) were achieved by 91.5% of the younger adults and 76.0% of the older adults after the third vaccination. The potential for antibody-dependent enhancement of disease, which has been described for RRV in vitro, will need to be considered during the development of vaccines against RRV.[69,77]

Bunyaviruses

The Bunyaviridae are a large family of segmented, lipid-enveloped, negative-stranded RNA viruses. Vaccines against human pathogens representing two of the five genera, RVF virus (*Phlebovirus* genus) and Hantaan virus (*Hantavirus* genus), have been developed at Fort Detrick.

Rift Valley Fever Virus

RVF is a mosquito-borne infection endemic to sub-Saharan Africa and primarily affecting ruminant animals. Under appropriate climatologic conditions, however, explosive epizootics among animals and epidemics in humans occur with considerable morbidity and mortality. In recent years, RVF has demonstrated its ability to spread northward to Egypt and into Yemen and Saudi Arabia on the Asian continent. The consequences of further spread into naïve animal and human populations are potentially devastating.[78] The spread of West Nile virus (WNV) has demonstrated that viruses once bounded by the Atlantic Ocean have the potential to overcome that barrier. Thus, the development of a vaccine or other countermeasures against the RVF virus is important for the protection of human and animal populations in Africa and the Middle East, U.S. military personnel involved in campaigns in those regions, and livestock and people in the United States and elsewhere.

The U.S. Army has developed two vaccines to combat this threat: an inactivated RVF vaccine (TSI-GSD 200) and, more recently, a live attenuated product (RVF MP-12; TSI-GSD 223). The Entebbe strain of RVF, isolated from a mosquito pool in Bwamba County, Uganda, is the source for the inactivated vaccine.[79] The virus was passaged 184 times in adult mice, followed by two passages in fetal rhesus lung (FRhL) cells to form the production seed. Although the original vaccine was produced in primary African green monkey kidney cells, the current vaccine lots are produced in FRhL cells.[80,81] The vaccine is inactivated in 0.05% formalin. Following verification of viral inactivation, the residual formaldehyde is neutralized with sodium bisulfite to less than 0.01%. A study of the immunogenicity and safety of inactivated RVF vaccine in humans during a 12-year period showed that the vaccine was safe and immunogenic when the three-dose primary series and one booster are administered.[82,83] In particular, 540 (90%) of 598 volunteers given three 1.0-mL doses of TSI-GSD 200 subcutaneously on days 0, 7, and 28 responded with a $PRNT_{80}$ titer of 1:40 or greater. Three-fourths of the initial nonresponders developed a $PRNT_{80}$ titer of 1:40 or greater after a single booster. However, approximately 10% of recipients of the inactivated RVF vaccine require repeated boosting. In these individuals, a booster typically results in an adequate titer, which wanes over the next year to <1:40, prompting another booster (P.R. Pittman, unpublished data).

An isolated RVF strain recovered from a nonfatal human case that occurred during the first Egyptian epidemic in 1977 was used to derive the live attenuated RVF vaccine. The virus (ZH548) was passaged twice in suckling mouse brain, then once in FRhL cells. It was then attenuated by 12 serial alternating passages in human lung cell cultures (MRC-5 cells, certified for vaccine use) by previously described methods[84] in the presence or absence of 5-fluorouracil. The resulting RVF MP-12 vaccine is a lyophilized product originating from supernatant fluids harvested from the final mutagenesis passage. The vaccine has undergone extensive safety testing and challenge studies in several animal species: rodents, sheep (including pregnant ewes and naïve neonatal lambs), cattle (including in utero–vaccinated bovids), and monkeys.[85–89] Furthermore, Miller and colleagues[90] showed that RVF MP-12 vaccine, administered to sheep, results in long-lasting immunity and has limited potential to be transmitted to mosquitoes feeding on vaccinated animals.

RVF MP-12 has undergone clinical evaluation in human volunteers at USAMRIID. In a Phase I randomized, double-blind, dose-escalation/route-seeking study, 56 healthy, nonpregnant subjects were randomly selected to receive RVF MP-12 ($10^{4.7}$ plaque-forming units [PFU] subcutaneously, $n = 10$; $10^{3.4}$ PFU intramuscularly, $n = 6$; or $10^{4.4}$ PFU intramuscularly, $n = 27$) or placebo ($n = 13$).[91] Only infrequent and minor side effects were seen among placebo and MP-12 recipients. One volunteer had a titer of 1.3 log by direct

plaquing in cell culture. Six vaccinees had transient low-titer viremia detected by amplification only; all six of these volunteers were from the group receiving $10^{4.4}$ PFU intramuscularly. Neutralizing antibodies (measured by $PRNT_{80}$ titer), as well as RVF-specific immunoglobulin (Ig) M and IgG, were observed in 40 (93%) of 43 vaccine recipients. The highest peak geometric mean antibody titers were observed in the group receiving $10^{4.4}$ PFU intramuscularly. Overall, 28 (82%) of 34 RVF MP-12 recipients available for testing remained seropositive ($PRNT_{80}$ = 1:20) at 1 year following inoculation.[91]

A joint program between the University of Texas Medical Branch and USAMRIID for further development of the RVF MP-12 vaccine has been completed. This study, funded by the National Institute of Allergy and Infectious Diseases, involved the administration of a single intramuscular dose of RVF MP-12 vaccine to 19 naïve male and nonpregnant female subjects. The vaccine was safe and immunogenic in this vaccine trial. A total of 18 (95%) of 19 subjects developed neutralizing antibodies against RVF virus, as determined by a $PRNT_{80}$ titer of 1:20 or more. Results suggested that the vaccine resulted in, at most, only low-level viremia. No virus was detected by direct plaque assay; however, during the first 14 days after vaccination, nine MP-12 isolates were recovered from five subjects with the use of amplification by blind, double passage in Vero cells. No single-nucleotide polymorphisms or reversions were observed in the attenuating mutations of the parent virus.[92]

Another RVF live attenuated vaccine candidate is Clone 13. This vaccine candidate is a plaque-purified clone of RVF virus that contains a large deletion in the small (S) genome segment that disrupts the biological functions of the nonstructural proteins NSs. Clone 13 has proven highly immunogenic in mice, sheep, and goats, although it has demonstrated only moderate immunogenicity in cattle.[93,94]

Other approaches to the development of vaccines against RVF virus, all of which are at the preclinical stage, include vaccines based on viral vectors[95–97]; DNA vaccines with molecular adjuvants[98]; subunit vaccines based on purified proteins[99–103]; and vaccines based on single-cycle replicable vaccine mutants.[104]

Hantaviruses

Hantaviruses causing hemorrhagic fever with renal syndrome (HFRS) have been, and continue to be, significant endemic disease threats to U.S. military forces on the Korean peninsula and throughout Europe.[105] At least 14 distinct viral strains of HFRS-causing viruses are distributed worldwide.[106] Three viral proteins are able to induce protection: two surface glycoproteins, G1 and G2 (also called Gn and Gc), and the N nucleocapsid protein. Neutralizing antibodies are protective, although T-cell responses may also be useful.[107] In addition to HFRS, hantaviruses are responsible for hantavirus pulmonary syndrome.[106,108] The hantavirus causing this syndrome in the southwest United States is called Sin Nombre virus (SNV). Humans become infected with hantaviruses via inhalation of aerosolized rodent excreta.[109] The prototype hantavirus, Hantaan, was first isolated in Korea, and the first vaccine also was developed there to protect against HFRS.[108,110,111] This inactivated vaccine, the ROK84/105 strain, harvested from suckling mouse brains, is concentrated by protamine sulfate precipitation and centrifugation. The concentrate is then exposed to formalin inactivation and purified by ultrafiltration and sucrose gradient ultracentrifugation. Aluminum hydroxide is added to the vaccine as an adjuvant, thimerosal as a preservative, and gelatin as a stabilizer. The final product, produced by Rhein Biotech, reportedly has less than 0.01 ng/mL of myelin basic protein. The recommended regimen for this product, Hantavax, is two doses of 5120 ELISA units (0.5 mL) given 1 month apart by the subcutaneous or intramuscular route.

Little has been published on the vaccine, but the manufacturer reports that tolerance is good, although allergic reactions occur, presumably as a result of mouse brain antigens. A serologic response measurable by indirect fluorescence is seen in nearly all vaccinees. Neutralizing antibodies are usually absent after one dose, but are measured in approximately 75% of subjects after the second dose.[112] They decrease to 16% at 12 months, at which time a booster is recommended. Although placebo-controlled data on the efficacy of the vaccine are not available, a similar vaccine made in North Korea reportedly showed 88% to 100% efficacy, and Hantavax itself has been effective in uncontrolled epidemiologic studies done in South Korea and Yugoslavia.[113,114] Although neutralizing antibody responses were not persistent and not stimulated by a booster dose,[107,115] a case-control study conducted in the Republic of Korea estimated efficacies of 46% for two doses and 75% for three doses, but with wide confidence limits.[116]

Vaccines made in cell culture against hantavirus and Seoul virus strains are licensed in China. The substrate is cell cultures of golden hamster kidney or Mongolian gerbil kidney. The efficacy of these bivalent vaccines is reportedly better than 90%, with fewer reactions than after the use of mouse brain–derived vaccine.[113,114]

Another Korean company has developed a hantavirus vaccine made in Vero cells that protected challenged animals by eliciting strong responses to the G1, G2, and N proteins of the virus.[117]

At USAMRIID, advances in technology fueled the development of bioengineered hantavirus vaccines. Two experimental approaches included recombinant vaccinia-vectored Hantaan virus vaccine carrying envelope and nucleocapsid genes[118,119] and the same genes inserted into bacterial plasmids as DNA vaccines. The recombinant vaccinia-vectored Hantaan virus vaccine was efficacious in the hamster infectivity model; even if preexisting immunity to vaccinia virus was present, it could be overcome by a second intramuscular injection of the vaccine candidate.[118] A double-blind, placebo-controlled clinical trial involving 142 volunteers using two subcutaneous injections 4 weeks apart showed that, for vaccinia-naïve volunteers, neutralizing antibodies to Hantaan virus or vaccinia virus were detected in 72% or 98%, respectively, whereas neutralizing antibodies to Hantaan virus were detected in only 26% of vaccinia-immune volunteers, showing the effect of prior vector-directed immunity.[119] This vaccine has been abandoned.

DNA plasmids coding for the G1 and G2 proteins protected monkeys against challenge with a South American hantavirus and elicited antibodies that exerted passive protection in hamsters.[120] Boosting the monkeys 1 to 2 years later showed that immunological memory had been induced.[121] Alphavirus replicons, baculovirus-produced proteins, and chimeric hepatitis B VLPs are all under study as additional hantavirus vaccine candidates.[113,122,123]

Boudreau and colleagues[124] report on a Phase I clinical trial that evaluated Hantaan virus and Puumala virus M-segment DNA vaccines for preventing HFRS. Each volunteer received either Hantaan DNA vaccine (n = 9), Puumala DNA vaccine (n = 9), or both vaccines (n = 9). Three doses (containing 8 μg DNA/4 mg gold per dose), administered 4 weeks apart, were provided to volunteers by particle-mediated epidermal delivery. The single vaccines elicited neutralizing antibodies to Hantaan virus or Puumala virus, respectively, in 30% and 44% of vaccinees. Neutralizing antibodies to one or

both viruses were detected in 56% of volunteers who received the combined vaccine. In an attempt to increase seroconversion, Hooper and colleagues[125] conducted a Phase I clinical trial of these two DNA vaccines delivered by intramuscular electroporation, similar to the Boudreau study. Volunteers (three groups of nine individuals each) received three doses, 28 days apart, of either the Hantaan DNA vaccine, the Puumala DNA vaccine, or a mixture of both vaccines. Each dose of vaccine contained 2 mg DNA in a total volume of 1 mL saline; the combined vaccine contained 1 mg of each DNA vaccine. Neutralizing antibodies were found in 56% and 78% of volunteers who received the Hantaan or Puumala DNA vaccines, respectively. Results of the combined vaccine showed that 78% of vaccinees developed neutralizing antibodies against Puumala virus. The three volunteers with the highest antibody levels against Puumala also developed neutralizing antibodies against Hantaan. No serious adverse events resulting from the vaccines were noted in either study.

Hooper and colleagues[126,127] also found that DNA vaccines encoding the virus envelope glycoproteins of SNV or Andes virus (both of which cause hantavirus pulmonary syndrome) elicit high-titer neutralizing antibodies in animal models. Hamsters vaccinated with three doses of the SNV DNA vaccine were fully protected from lethal challenge with SNV. Further, a pan-hantavirus vaccine using Andes virus, SNV, Hantaan virus, and Puumala virus plasmids elicited neutralizing antibodies against all four viruses.[126]

West Nile Virus

The spread of the flavivirus WNV from its introduction into New York City in 1999 across the United States has been a dramatic example of an emerging infection. WNV is an arbovirus of birds, for which it is often lethal, and is transmitted by multiple mosquito species. Although the incidence of human infections has recently decreased, WNV frequently causes febrile illness, with the complication of meningoencephalitis in about 1% of infections.[128]

WN-VAX, a formalin-inactivated WNV vaccine derived from a strain isolated in New York City in 1999, has been tested in a number of animal models. This vaccine, administered intraperitoneally in two doses, protected 100% of 4-week-old mice against a lethal challenge with WNV.[129] More recently, Muraki and colleagues[130] found that mice passively immunized with serum from WN-VAX–immunized mice were also protected from lethal WNV infection. When administered to macaques, WN-VAX elicited neutralizing antibodies.[130] Clinical trials on this vaccine are planned.

Several Phase I and Phase II clinical trials have been completed on WNV vaccine candidates. The ChimeriVax West Nile vaccine candidate, containing a yellow fever virus backbone and expressing the premembrane and envelope proteins of WNV, was reported to be safe and induced neutralizing antibodies in a Phase I clinical trial.[131,132] In a Phase II clinical trial, ChimeriVax was well tolerated, and more than 96% of vaccinees seroconverted.[133]

In a Phase I clinical trial, a DNA vaccine candidate elicited neutralizing antibodies against WNV.[134] A recombinant subunit vaccine candidate, consisting of an envelope protein truncated at the C-terminal end and containing 80% of the N-terminal amino acids of the native WNV protein (WN-80E) mixed with adjuvant, produced antibodies detectable by ELISA and neutralizing antibody assays. In the challenge experiment, all control rhesus macaques had detectable viremia for at least 3 days after challenge, whereas none of the vaccinated animals showed viremia.[135] A Phase I trial assessing the safety of this vaccine was recently completed.

Arenaviruses

Several members of the *Arenaviridae* family of segmented, negative-stranded RNA viruses are recognized as causative agents for viral hemorrhagic fever syndromes in humans. To date, efforts to develop protective immunogens against arenaviruses have met with limited success.[136] During the 1980s, the first successful vaccine against an arenavirus, Junin virus, was developed through a collaboration between the government of Argentina and the U.S. Army. Efforts to develop vaccines against other pathogenic arenaviruses have resulted in a number of promising candidates, but none of these has progressed to clinical trials.[137]

Junin Virus

Junin virus is the causative agent of AHF, which is endemic to the pampas of north-central Argentina. Humans become infected with the Junin virus by inhalation of infected rodent secretions and excretions.[138–140] Death occurs in 15% to 30% of untreated patients afflicted with AHF.

For several decades, attempts were made in Argentina to develop an efficacious vaccine against AHF. The resulting inactivated and live attenuated vaccine candidates all failed for various reasons.[136] The product developed at USAMRIID, Candid #1, is a descendant of the prototype XJ strain Junin virus, isolated in guinea pig from a fatal AHF case. Following another passage in the guinea pig, the virus underwent 44 newborn mouse brain passages, then was cloned and passaged 19 times in certified FRhL cells.[136,141,142] Candid #1 proved effective in preventing disease in guinea pigs and rhesus macaques after lethal Junin virus challenge.[140,143,144] In Phase I and Phase II clinical testing in humans, Candid #1 was safe and immunogenic.[145,146] More than 90% of volunteers developed antibodies against Junin virus, although at lower levels than those seen after mild natural infection,[147] and 99% developed a Junin virus–specific cellular immune response. In a pivotal efficacy study, 3255 volunteers were randomized to receive the vaccine and 3245 were randomized to receive a placebo. During the trial, 23 volunteers developed an illness that met the clinical case definition for AHF.[148,149] Of these, 22 had received a placebo and 1 had received the vaccine; vaccine efficacy by intent-to-treat analysis was 95% (95% confidence interval, 82%–99%; $P < .001$). Argentina's National Institute of Human Viral Diseases produced its own Junin vaccine, also called Candid #1, against AHF and tested it in 946 human volunteers to compare its safety and immunogenicity with that of the Candid #1 vaccine produced in the United States and used in previous studies.[150] The vaccine was comparable to the U.S.-produced vaccine in terms of safety and efficacy. No severe adverse events were related to the vaccine. As a result of this study, Argentina's national regulatory authority (ANMAT) licensed the locally produced Candid #1 vaccine.[151,152]

Vaccination with Candid #1 is recommended for persons at risk of occupational (agricultural or laboratory) exposure to the Junin virus. The development of this vaccine represents a successful collaboration between USAMRIID and the Argentine Ministry of Health and Social Action, under the auspices of the United Nations Development Programme and the Pan American Health Organization. Over the years, hundreds of thousands of people in Argentina and other countries have been inoculated, with an excellent record of safety and effectiveness.[153]

Lassa Virus

Another important Arenavirus is Lassa virus, transmitted by rodents in West Africa, where there has been a recent large

epidemic. Several candidate vaccines have shown protection in nonhuman primate models, including reassortants, vesicular stomatitis virus-vectored recombinants, and alphavirus replicons.[153a]

Middle East Respiratory Syndrome Virus

The Middle East respiratory syndrome (MERS) virus—like the severe acute respiratory syndrome (SARS) virus that spread from China and caused a worldwide outbreak in 2002–2003—is a coronavirus.[154,155] The reservoir for MERS is camels, particularly young animals, which acquire a respiratory illness that can be transmitted to humans.[156,157] In addition, human-to-human transmission has been repeatedly observed in Saudi Arabia and in South Korea.[158] All coronaviruses possess a glycoprotein spike that induces neutralizing antibodies; both monoclonal antibodies and vaccines based on the spike have shown good protection in experimental challenge models, including macaques.[159] Development of vaccines for camels and for humans is proceeding.

Novavax has developed 0.2-μm nanoparticles consisting of MERS spike protein which, together with ISCOMATRIX adjuvant, have been shown to induce neutralizing antibodies in mice.[160] German workers have used the MVA vaccinia virus as a vector for the MERS spike protein and induced neutralizing antibodies and protection in a mouse challenge model.[161] The Vaccine Research Center at the National Institutes of Health has developed plasmids and recombinant proteins representing the full-length MERS spike or the S1 domain of the spike. When both antigens were administered to mice or macaques, neutralizing antibodies were induced and animals were protected from challenge with the virus.[162] The further development of these candidate vaccines will depend on epidemiology and market size.

LIMITED-USE VACCINES AGAINST BACTERIAL DISEASES

Q Fever

Q fever is a highly infectious zoonotic disease of humans usually caused by aerosol transmission of *C. burnetii* from infected sheep or goats.[163] The organism was thought to be a *Rickettsia* but is now considered to be related to *Legionella* bacteria. Fever and pneumonia are the most frequent clinical manifestations of Q fever, although hepatitis, endocarditis, and a variety of other complications may develop.[164] Antibiotics are effective but may act slowly, and chronic fatigue syndrome after Q fever has been reported, presumably as a result of cytokine dysregulation.[165]

Mechanisms of immunity to Q fever are complex. Although antibodies are important to clear extracellular organisms, it is sensitization of T-cell lymphocytes to Q fever antigen and secretion of lymphokines that clear intracellular infection and provide immunologic memory.[166]

In the development of a Q fever vaccine, *C. burnetii* was adapted to grow in the chick embryo yolk sac, and an early vaccine was made by formalin inactivation of organisms produced in eggs.[167] This vaccine was effective in humans, including in those subjected to experimental challenge, but it also occasionally resulted in severe local reactions that sometimes progressed to abscess formation. Eventually it was shown that reactions were associated with preexisting immunity to Q fever. Accordingly, the practice of screening prospective vaccinees was adopted—using serology for Q fever antibodies and observation for local induration after inoculation with a skin test antigen made from diluted vaccine.

Advances in *Coxiella* biology and vaccinology led to a new generation of vaccines. For example, evidence showed that only Phase I Q fever organisms, analogous to smooth forms of bacteria, were protective, and that a transition to Phase II (rough) would occur if the organism was passaged too many times in chick embryo. Therefore, Q fever vaccines are based on Phase I organisms only.[168] In addition, researchers recognized that purification to remove chicken protein and lipid and isolation of whole inactivated *Coxiella* by extraction, filtration, or centrifugation would result in a cleaner, less reactogenic, and highly immunogenic product. Two whole-cell vaccines came into use in high-risk subjects: an Australian vaccine made and licensed by Commonwealth Serum Laboratories[166,169] and a vaccine (IND 610) made and tested by the U.S. Army.[170] Both are administered as a single 30-μg subcutaneous injection. The Australian vaccine, Q-Vax, is purified using high concentrations of NaCl to remove nonprotective antigens. The U.S. Army vaccine, Whole-Cell, Inactivated, Q-Fever Vaccine NDBR 105, IND 610, is also extracted with NaCl, then subjected to ethanol–Freon 113 extraction; finally, it is purified further on a $CaHPO_4$ $2H_2O$ (brushite) column. Despite these purification processes, however, skin testing of prospective recipients remains necessary to prevent serious reactions in persons who have experienced prior infection.

Whole-cell vaccines have undergone considerable clinical testing. In Australia, a placebo-controlled trial[171] and an open trial in abattoir workers[172,173] showed efficacy approaching 100% (Table 12.3). From 2002 to 2006, during a vaccination program funded by the Australian government, the incidence of Q fever in Australia declined by more than 50%.[166,174] The U.S. Army vaccine was subjected to a controlled challenge in volunteers; this trial also proved highly successful.[175] Two meta-analyses found that Q-Vax was, respectively, 97% and 83% to 100% effective, but the data were considered to be of only moderate quality.[176,177] A Q fever outbreak, apparently caused by dairy goats, occurred in The Netherlands between 2007 and 2011, affecting more than 4000 individuals.[178] Control measures that included Q-Vax vaccination of individuals at high risk for developing chronic Q fever were

TABLE 12.3 Protection by Q Fever Vaccines

Vaccine	Type of Study	No. of Cases of Q Fever		
		Vaccinated	Unvaccinated	Protection (%)
United States	Random, challenge	2/32	5/6	92
Australian	Random, placebo-controlled	0/98	7/102	100
Australian	Observational	3[a]/2716	52/2012	96–100

[a]Vaccinated during incubation period of Q fever.

Data from Ormsbee RA, Marmion BP. Prevention of Coxiella burnetii *infection: vaccines and guidelines for those at risk. In: Marne TJ, ed.* Q Fever: The Disease, *vol 1. Boca Raton, FL: CRC Press; 1990:225–248.*

effective in containing the outbreak despite limited coverage of the vaccination campaign.[179–181]

To eliminate the problem of reactions, third-generation acellular Q fever vaccines have been prepared in the United States[182,183] and in the former Czechoslovakia.[184] These vaccines, extracted with chloroform–methanol or other lipid solvents to remove lipid A (thought to be the chief offending substance in *C. burnetii*), have not yet undergone sufficient clinical testing to determine whether they are equivalent to whole-cell Q fever vaccine in protection of humans. In addition, it is not yet clear if these vaccines overcome the problem of serious reactions in preexposed individuals.[185,186]

A live attenuated Q fever vaccine produced in chick embryo yolk sac has been developed in Russia, but its safety has been questioned. Other vaccine approaches under investigation are based on the P1 29-kDa protein, a 67-kDa antigen, and DNA plasmids coding for various proteins.[187–189]

Better Q fever vaccines are needed that induce antibodies and T-helper type 1 (Th1) cell responses and yet are well tolerated by individuals with preexisting immunity. Elucidation of the key protective antigens is required.[185,189]

Tularemia

Tularemia, a bacterial bioterrorism threat, can be a significant endemic and epidemic human disease.[190] The causative bacterium of tularemia, *Francisella tularensis*, was isolated in 1912; in 1919, Edward Francis made the association with the human disease then known as "deer fly fever."[191]

Andersson and colleagues[192] describe the transcriptional response in peripheral blood following ulceroglandular tularemia in humans. The authors identified seven genes whose changes in expression predict the early phase of tularemia. In addition to identifying how the host defense develops or is turned off by *F. tularensis* invasion, these data are important in identifying potential diagnostic markers for tularemia infection.

Many of the more than 200 cases of *F. tularensis* documented in the American medical literature resulting from laboratory exposure occurred in laboratory personnel who had received one or more injections of a phenol-killed vaccine and/or acetone-prepared vaccine.[193–196] One of these early inactivated products, the Foshay vaccine, showed incomplete protective efficacy against tularemia organisms introduced by the respiratory and intracutaneous routes.[197,198] Although circulating antibodies could be demonstrated following administration of these vaccines, the antibodies generated were not protective. It was concluded that the protective antigen of *F. tularensis* was destroyed in the inactivation procedures used to prepare the vaccines, although now antibodies alone are considered insufficient.

Partly as a result of the experience with killed vaccines, an effort to develop a live vaccine against *F. tularensis* was undertaken. By avoiding destruction of the protective antigen, live vaccines, which cause actual infection, are thought to produce an immunity closer to that caused by the disease itself, in particular by generating persistent antibodies and CD8-mediated cellular immunity, both of which are required for protection against disease.

Soviet investigators initially developed a live tularemia vaccine in 1942.[199] Ampoules of this "viable" tularemia vaccine were brought to the United States from the Russian Institute of Epidemiology and Microbiology (Gamaleia Institute) by Shope[200] in 1956. From an ampoule of this product, Eigelsbach and Downs[201] derived a vaccine strain, which they designated LVS.

Studies done at Fort Detrick in the early 1960s showed that LVS was protective for mice and guinea pigs after a challenge.[201] This protection was subsequently found to be the result of cell-mediated immunity when passively transferred spleen cells from immunized mice provided protection to nonimmune recipients.[202] Similar immunization studies were done in monkeys, which also demonstrated a significant immune response to vaccination with LVS.[203]

LVS initially was given by scarification to volunteers at Fort Detrick in 1958.[204] The vaccination procedure and adverse events after vaccination were evaluated in 29 subjects.[198] Only a pink scar remained 1 month after vaccination. Transient axillary lymphadenitis was observed in approximately half of the subjects, but none of the 29 men exhibited fever or other systemic reactions following immunization with LVS. All persons showed bacterial agglutinin antibodies; peak titers were observed 29 to 59 days after vaccination and were sustained.[205]

After challenge doses of up to 2500 organisms, a significant protective effect was seen in the volunteers vaccinated with LVS, with only 20% of vaccinated persons showing clinical illness compared with 85% of the unvaccinated control subjects. None of the vaccinated persons developed symptoms severe enough to require treatment. At higher challenge doses, however, immunity was overcome; after challenge with 25,000 organisms, 90% of vaccinated persons showed some symptomatology compared with 85% of the control subjects. However, LVS modulated illness severity such that only 60% of vaccinated volunteers had symptoms severe enough to require treatment, compared with 100% of those in the control group.[205]

The efficacy of the live tularemia vaccine, when administered by aerosol, has also been evaluated.[206,207] In one study,[207] aerosol vaccination with LVS protected 6 of 16 subjects against challenge with the virulent SCHU-S4 strain of *F. tularensis*. All protected subjects had measurable circulating antibodies, while only one of the 10 subjects who developed disease had circulating antibodies.

LVS (also referred to as NDBR 101) has been used since the mid-1960s and has been associated with a significant decline in the rate of laboratory-acquired infections at Fort Detrick.[208] In the absence of firm knowledge concerning the attenuating mutations in LVS and its residual virulence, the vaccine remains investigational and is administered only under protocol and with written informed consent. The lots undergo lot-release and potency tests as required by FDA for IND products.

Efforts are underway to develop new-generation vaccines against tularemia using modern technologies. Lipopolysaccharide from *F. tularensis* gives partial protection through the induction of antibodies, but clearly, cell-mediated responses to other antigens will be necessary for an optimal vaccine against highly virulent strains.[209,210] In addition, aerosol delivery may give better protection than peripheral inoculation.[211] Much work is underway to create new live attenuated mutants[211–217] and novel subunit vaccines.[218] In one promising approach, the oxidant-sensitive *emrA1* mutant of *F. tularensis* LVS elicited a strong humoral immune response in mice without causing adverse effects. Mice vaccinated with a single intranasal dose of the *emrA1* mutant of *F. tularensis* LVS were protected against a lethal respiratory LVS challenge; this vaccine also provided partial protection against lethal challenge with the virulent *F. tularensis* SchuS4 strain.[219]

Brucellosis

Primarily a disease of animals, brucellosis first was described in humans in 1859 as "Mediterranean gastric remittent fever" or "Malta fever."[220] The consumption of unpasteurized goat milk was found to be the source in the Malta epidemic.[182]

Bruce[221] isolated the etiologic agent in 1886 from a fatal case of brucellosis. Initially named *Micrococcus melitensis*, the genus later was named for its discoverer and is currently known as *Brucella melitensis*. *Brucella* species are non–spore-forming, Gram-negative coccobacilli that exist worldwide. Several animal species become infected with *Brucella* species. Humans become infected on contact with infected animals, by consuming unpasteurized infected milk or other dairy products, and by working with the organism under laboratory conditions. Brucellosis manifests with nonspecific flu-like symptoms that are often much more prominent than the gastrointestinal symptoms.[222] The feasibility of infection by aerosol makes *Brucella* a possible agent of biowarfare.

Effective vaccines, both live and killed, are available for animals. Use of these vaccines has led to decreases in human disease where animal infection is enzootic. Nevertheless, an estimated 500,000 annual human cases still occur worldwide.[223] A live vaccine, *Brucella abortus* S19, was extensively used in Russia and gave more than 50% protection, although it was somewhat reactogenic.[224]

Ongoing research seeks to further attenuate live vaccines used in animals and to develop subunit vaccines based on membrane and ribosomal proteins or DNA plasmids coding for those proteins.[225–234] For example, protection in animals has been achieved with an outer membrane protein and its gene,[235,236] with periplasmic protein bp26,[237] by intranasal administration of *Brucella* lipopolysaccharide complexed with *Neisseria meningitidis* outer membrane protein,[238,239] and a recombinant glucokinase protein *Brucella* vaccine candidate.[240]

Pseudomonas

Pseudomonas aeruginosa is a common pathogen in ventilated patients and in those with burns; however, it also causes life-threatening infections in people with cystic fibrosis owing to its production of a thick, mucoid capsule. Although antibodies to the lipopolysaccharide develop in people with cystic fibrosis after infection, they are of low affinity and do not protect. In contrast, artificial immunization with lipopolysaccharide from eight different strains coupled chemically with the *Pseudomonas* exotoxin A elicited high-affinity IgG antibodies.[241,242] Studies of cellular immune responses showed induction of antigen-specific lymphocyte proliferation and a Th1-like cytokine secretion.[243] During a 10-year period, people with cystic fibrosis immunized with O polysaccharide conjugated to toxin A had fewer *Pseudomonas* infections and a tendency toward less colonization with mucoid strains (Herzog C. Personal communication to S. Plotkin, November 26, 2002).[244,245] Importantly, lung function was also better in vaccinees.[244,246,247] In a Phase III trial, patients with cystic fibrosis who received four doses of a bivalent *P. aeruginosa* flagella vaccine were significantly less likely to have one or more acute *P. aeruginosa* infections than were those who received placebo (19.6% vs 30.7% among vaccinees and placebo recipients, respectively).[248] However, additional antigens are expected to provide better protection.[249] A vaccine containing formalin-inactivated whole *Pseudomonas* bacteria has been administered orally to healthy volunteers without adverse reactions and with significant serum IgA responses.[250]

Many other experimental vaccines are under development, including ones based on flagellar antigens, outer membrane proteins such as OprF and OprI, and exotoxin A.[251–254] For example, one DNA vaccine encoding *Pseudomonas* exotoxin A and PcrV has shown promise in mice,[255] and a *P. aeruginosa* hybrid outer membrane protein OprE/I (IC43) vaccine was well tolerated and immunogenic in a recent randomized, placebo-controlled Phase I study.[256] The mucoid exopolysaccharide of *P. aeruginosa* has been conjugated with keyhole limpet hemocyanin and found to elicit opsonophagocytic antibodies in mice.[257] An attenuated salmonella vector expressing the O antigen of *P. aeruginosa* lipopolysaccharide given intranasally protected mice against challenge.[258] Interestingly, Th17 responses, as in the case of other mucosal bacteria, may be necessary for protection,[259] and mucosal routes of administration may improve efficacy.[260–262]

The virulence factor alginate is produced by mucoid forms of *P. aeruginosa*. One strategy in *Pseudomonas* vaccine development is to raise opsonic antibodies against alginate. One group conjugated alginate to the outer membrane vesicle of *N. meningitides*. Immunization of mice with this conjugate provided protection against intranasal challenge with *P. aeruginosa*,[263] thus validating the concept.

Helicobacter pylori

Helicobacter pylori, which colonizes the stomach early in life but exerts its effects in adults, is the cause of gastritis, peptic ulcers, and low-grade gastric lymphomas.[264,265] The mechanisms of disease involve stimulation of Th1, regulatory T, and possibly Th17 cells; antibody and cellular mechanisms seem to be involved in the protection demonstrated in animal models. Clinical trials have tested urease, which is secreted by *H. pylori*; the cagA pathogenicity island; the VacA cytotoxin; and the neutrophil-activating protein.[266] The results in the past have not been promising; more recently, however, an aluminum-adjuvanted combination of the latter three antigens stimulated strong antibody and cellular responses in volunteers and will undergo further clinical development.[267] Other approaches, such as VLPs,[268] epitope-based vaccines,[269–271] alkyl hydroperoxide reductase and mannosylated AhpC,[272] and thiolperoxidase,[273] have shown promise in single- and multicomponent vaccine studies using murine models.

Botulism

Botulism is a neurologic intoxication caused by botulinum neurotoxin, the most poisonous agent known. It is produced by the bacterium *Clostridium botulinum*. Seven toxin types (A through G) have been recognized; all but G cause human disease.[274] Botulinum neurotoxin is a significant biowarfare and bioterrorism threat. The neurotoxin binds to the presynaptic membrane and prevents acetylcholine release, resulting in a symmetric, descending flaccid paralysis with classical bulbar palsies characterized by diplopia, dysphonia, dysarthria, and dysphagia.[275] As paralysis progresses, generalized weakness occurs; if untreated, death occurs from airway obstruction and diaphragmatic muscle paralysis. Interestingly, this toxin has many therapeutic uses as well—notably, but not limited to, treatment of blepharospasm, strabismus, cervical torticollis, and various dystonias.[276–278]

Pentavalent (ABCDE) botulinum toxoid (PBT) was available as a prophylactic countermeasure to botulism from 1959 until November 30, 2011, when the Centers for Disease Control and Prevention stopped providing the vaccine under IND 161 because of reduced potency and increased reactogenicity.[3] Although the old methodology for producing toxoids (i.e., the methods used to produce PBT) can be improved by reducing formalin concentrations and increasing the purity of the botulinum proteins, the most advanced candidates are recombinant nontoxic proteins. The Hc subunits of toxin types A and B were immunogenic in a clinical trial and are proceeding in development.[279–282] Other peptides of the toxin, including the light chain and the N terminal of the heavy chain, are also being tested[283] (Fig. 12.1). A nonreplicating

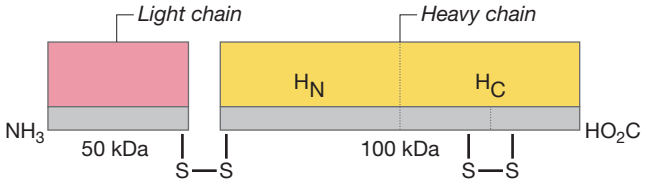

Figure 12.1. Structure of botulinum toxin.

adenovirus-vectored monovalent (C) vaccine, given intranasally or orally, has also been shown to induce serum and secretory antibodies and to protect animals.[284,285] A recombinant bivalent vaccine (rBV A/B) has completed Phase II safety and immunogenicity testing in humans.[286] Inevitably, new vaccines against botulism will have to be licensed through a demonstration of efficacy in animals. Although a titer of antibodies correlating with efficacy is not known for humans, studies in guinea pigs suggest that very low levels (<0.1 U) will be protective.[287]

In March 2013, the FDA approved a heptavalent equine (ABCDEFG) botulism antitoxin to treat individuals with symptoms of botulism after exposure or suspected exposure to botulinum neurotoxin.[288,289] BabyBIG has been available in California to treat infant botulism since 2003.[290] However, no vaccines for prophylactic protection against botulism are currently available in the United States.

Clostridium difficile

Clostridium difficile is an inhabitant of the human gastrointestinal tract that, under certain conditions, produces toxins that cause diarrhea. The precipitating cause of *C. difficile* infection (CDI) seems to be the elimination of other intestinal flora by antibiotic treatment. Incidence of the disease has increased considerably in recent years.[291–294] Antibodies against both of the toxins produced by *C. difficile*, A and B, are needed to prevent illness.[295,296]

Much work is in progress to develop and use monoclonal antibodies against the toxins for prophylaxis and treatment.[297–300] Vaccines against CDI are currently under clinical development at Sanofi Pasteur[301] and Pfizer.[301a] Both candidate *C. difficile* toxoid vaccines are composed of a highly purified formalin or genetically inactivated toxoids together with aluminum adjuvants delivered by intramuscular injection. Early clinical data suggest a relationship between an immune response to toxins A and B with protection against a primary or recurrent case of CDI.[297,302–305] After colonization, a systemic anamnestic response to toxin A, as evidenced by increased serum IgG antitoxin A levels, is associated with asymptomatic carriage of *C. difficile.* Serum IgG antitoxin A levels have also been significantly correlated with IgG levels against toxin B antigens, which were also higher in asymptomatic carriers.[303] In patients with CDI, higher IgG antibodies to toxin A are associated with protection against recurrence.[303] In an initial Phase I study, the candidate vaccine induced IgG antibodies to toxins A and B with resolution of recurrent diarrhea.[304] In a more recent Phase II study, the Sanofi candidate, given in a high dose, elicited functional antibody responses in adult and elderly volunteers for at least 180 days without safety issues.[306]

In addition, work has begun on the use of colonization factors as vaccine antigens. These include a protease (Cwp84) that is an adhesion molecule[307,308] and a cell wall polysaccharide conjugated to the diphtheria toxoid variant CRM.[309] In another approach to vaccine development, Baliban and colleagues[310] created a synthetic DNA vaccine encoding receptor binding domains from *C. difficile* toxin A and toxin B. In mice and nonhuman primates, this vaccine demonstrated robust immunogenicity, and immunized mice were protected from a challenge with *C. difficile* spores from homologous and heterologous strains.

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