

Chapter 20

ALPHAVIRUS ENCEPHALITIDES

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INTRODUCTION

During the 1930s, three distinct but antigenically related viruses recovered from moribund horses were shown to be previously unrecognized agents of severe equine encephalitis. Western equine encephalitis virus (WEEV) was isolated in the San Joaquin Valley in California in 1930¹; Eastern equine encephalitis virus (EEEV) was isolated in Virginia and New Jersey in 1933^{2,3}; and Venezuelan equine encephalitis virus (VEEV) was isolated in the Guajira peninsula of Venezuela in 1938.⁴ By 1938, it was clear that EEEV and WEEV were also natural causes of encephalitis in humans,⁵⁻⁷ and naturally acquired human infections with VEEV occurred in Colombia in 1952 in association with an equine epizootic.⁸

Although these viruses cause similar clinical syndromes in horses, the consequences of the infections they cause in humans differ. Eastern equine encephalitis (EEE) is the most severe of the arboviral encephalitides, with case fatality rates of 30% to 70%, and neurological sequelae common in survivors.⁹ WEEV appears to be less neuroinvasive but has pathology similar to that of EEE in patients with encephalitis. In contrast, severe encephalitis resulting from VEEV is rare in humans except for children. In adults, VEEV usually causes an acute, febrile, and incapacitating disease with prolonged convalescence.

The three viruses are members of the *Alphavirus* genus of the family *Togaviridae*. As with most of the alphaviruses, VEEV, EEEV, and WEEV are transmitted by mosquitoes, and are maintained in enzootic cycles with various vertebrate hosts. Thus, the natural epidemiology of these viruses is controlled by environmental factors that affect the interactions of the relevant mosquito and reservoir host populations. Of the 31 viruses currently classified within this group, VEEV, EEEV, and WEEV are the only viruses regularly associated with encephalitis. Although these encephalitic viruses are restricted to the Americas, as a group, alphaviruses have worldwide distribution and include other epidemic human pathogens. Among those pathogens, chikungunya virus (Asia, Africa, and the Americas), Mayaro virus (South America), o'nyongnyong virus (Africa), Ross River virus (Australia and Oceania), and Sindbis virus (SINV; Africa, Europe, and Asia) can cause an acute febrile syndrome often associated with debilitating polyarthritic symptoms.

Although natural infections with the encephalitic alphaviruses are acquired by mosquito bite, these viruses are also highly infectious by aerosol. VEEV has caused more laboratory-acquired disease than any other arbovirus. Since its initial isolation, at least 150 symptomatic laboratory infections have been reported,

most of which were known or thought to be aerosol infections.¹⁰ Before vaccines were developed, most laboratories working with VEEV reported disease among their personnel. The ability of aerosolized EEEV and WEEV to infect humans is less certain, relying on anecdotal evidence and animal studies. EEEV and WEEV are less commonly studied in the laboratory than VEEV, which may explain the lower incidence of laboratory-acquired infections. Therefore, fewer human exposures have occurred or the infectious dose is higher resulting in fewer incidences.

Perhaps as a consequence of their adaptation to dissimilar hosts in nature, the alphaviruses replicate readily and generally to very high titers in a wide range of cell types and culture conditions. Virus titers of 1 billion infectious units per milliliter of culture medium are not unusual, and the viruses are stable in storage and in various laboratory procedures. Because they can be easily manipulated in the laboratory, these viruses have long served as model systems by which to study various aspects of virus replication, pathogenesis, induction of immune responses, and virus-vector relationships. As a result, the alphaviruses are well described and their characteristics well defined.^{9,11,12}

The designers of offensive biological warfare programs initiated before or during World War II¹³ recognized that the collective in vitro and in vivo characteristics of alphaviruses, especially the equine encephalitis viruses, lend themselves well to weaponization. Although other encephalitic viruses could be considered as potential weapons (eg, the tickborne encephalitis viruses), few possess as many of the required characteristics for strategic or tactical weapon development as the alphaviruses:

- These viruses can be produced in large amounts in inexpensive and unsophisticated systems.
- They are relatively stable and highly infectious for humans as aerosols.
- Strains are available that produce either incapacitating or lethal infections.
- The existence of multiple serotypes of VEEV, as well as the inherent difficulties of inducing efficient mucosal immunity, confound defensive vaccine development.

The equine encephalitis viruses remain as highly credible threats, and intentional release as a small-particle aerosol from a single airplane could be expected to infect a high percentage of individuals within an area

of at least 10,000 km². Furthermore, these viruses are readily amenable to genetic manipulation by modern recombinant DNA technology. This characteristic is

being used to develop safer and more effective vaccines,^{14,15} but, in theory, it could also be used to increase the weaponization potential of these viruses.

HISTORY AND SIGNIFICANCE

Descriptions of encephalitis epizootics in horses thought to have been caused by EEEV were recorded as early as 1831 in Massachusetts.¹⁶ However, it was not until the outbreaks of EEE in Delaware, Maryland, and Virginia in 1933 and 1934 that the virus was isolated, and not until a similar outbreak in North Carolina in 1935 that birds were suspected as the natural reservoir.¹⁷ The initial isolation of EEEV from a bird¹⁸ and from *Culiseta melanura* mosquitoes,¹⁸ the two major hosts of the EEEV natural cycle, were both reported in 1951. Outbreaks of EEEV have occurred in most eastern states and in southeastern Canada, but they have been concentrated along the eastern and Gulf coasts. Although only 270 cases of EEE in humans were reported between 1964 and 2010 (<http://www.cdc.gov/easternequineencephalitis/tech/epi.html>), the social and economic impact of this disease has been larger than expected because of the high case fatality rate, significant long-term sequelae among survivors, equine losses, extreme concern among individuals living in endemic areas during outbreaks, and the surveillance and mosquito-control measures required. Isolation of EEEV from *Aedes albopictus* mosquitoes, which are prevalent in EEE endemic areas in the United States, has heightened concern because the opportunistic feeding behavior of these mosquitoes and their apparent high vector competence for EEEV suggest that they may be efficient bridge vectors for spillover infections of humans.¹⁹

The initial isolation in 1930 of WEEV from the brain tissues of a horse with encephalitis was made in the midst of a large epizootic in California, which involved at least 6,000 horses and with an approximate mortality of 50%.¹ Cases of human encephalitis in California were not linked to WEEV until 1938, when the virus was isolated from the brain of a child. During the 1930s and 1940s, several other extensive epizootics occurred in western and north-central states, as well as in Saskatchewan and Manitoba in Canada, which affected large numbers of equids and humans. For example, it has been estimated that during 1937 and 1938, more than 300,000 equids were infected in the United States, and in Saskatchewan, 52,500 horse infections resulted in 15,000 deaths.^{20,21} Unusually high numbers of human cases were reported in 1941: 1,094 in Canada and 2,242 in the United States. The attack rate in these epidemics ranged from 22.9 to 171.5 per 100,000, with case fatality rates of 8% to 15%.²¹

In the early 1940s, workers isolated WEEV from *Culex tarsalis* mosquitoes²² and demonstrated the presence of specific antibodies to WEEV in birds,²³ suggesting that birds are the reservoirs of the virus in nature. The annual incidence of disease in both equids and humans continues to vary widely, which is expected of an arthropodborne disease, and significant epidemics occurred in 1952, 1958, 1965, and 1975.²¹

VEEV was initially isolated during investigations of an epizootic occurring in horses in Venezuela in 1936, and the isolate was shown to be antigenically different from the EEEV and WEEV isolated previously in the United States.^{4,24} Over the following 30 years, many VEEV outbreaks were reported among horses, and humans became infected in large numbers in association with these epizootics.²⁵ Most of those infected recovered after suffering an acute, febrile episode, but abortions and stillbirths were observed in pregnant women and severe disease with encephalitis and death also occurred, mostly in children and older individuals. In the 1960s, major epizootics occurred in Venezuela, Colombia, Peru, and Ecuador, and spread to Central America in 1969.²⁶ These epizootics and previous ones were associated with significant human suffering, especially among rural people, who suffered not only from disease, but also from the loss of their equids, which were essential for transportation and agriculture. Between 1969 and 1971, epizootics were reported in essentially all of Central America and subsequently continued north to Mexico and into Texas. The most recent major epizootic occurred in Venezuela and Colombia in 1995.²⁷

Between active epizootics it was not possible to isolate the equine virulent viruses. During the 1950s and 1960s, however, several other attenuated, antigenically different VEEV strains were isolated from different geographical areas. These enzootic strains could be differentiated antigenically not only among themselves but also from the epizootic strains.²⁸ Enzootic strains used different mosquito vectors than the epizootic strains,²⁹ and most used rodents as reservoir hosts.³⁰ However, despite apparent avirulence for equids, at least some of the enzootic strains caused human disease.³¹

Laboratory studies with EEEV, WEEV, and VEEV quickly and often inadvertently demonstrated how easily these viruses could cause disease when inhaled. In 1943, eight cases of VEE in laboratory personnel resulted from aerosolization of the virus from contaminated

animal caging.³² In 1959, two reports from the former Soviet Union detailed an incident in which nine vials of VEEV were dropped in a stairwell infecting 24 people, including people who worked on adjacent floors.^{33,34} Before the vaccine and more sophisticated personal protective measures were developed, VEEV was among the most common laboratory-acquired infections.³⁵ It was primarily because of laboratory-acquired infections that it was realized that the current investigational new drug (IND) vaccines may not protect well against aerosol exposure to enzootic strains of VEEV.^{36,37} Two fatal laboratory accidents involving WEEV were reported in the late 1930s, one involving a centrifuge accident and another in which the route of infection was unknown.^{38,39} Other reports of laboratory-acquired infection of WEEV were not fatal.^{40,41} Although EEEV is considered the most virulent of the encephalitic alphaviruses, before 1967 only two cases of laboratory-acquired EEE occurred, neither of which was fatal.³⁵ Experimental studies in mice, hamsters, rats, and nonhuman primates (NHPs) have all corroborated the disease potential of VEEV, WEEV, and EEEV when aerosolized and inhaled.⁴²

Therefore, within 30 years of the initial isolation of EEEV, WEEV, and VEEV, an essentially accurate picture had emerged with respect to their endemic and epidemic behavior, arthropod vectors, reservoir hosts, potential for infection via the respiratory tract, and the diseases produced. Although not then understood at the molecular level, these three viruses were well described as agents of disease, and the basic methods for their manipulation and production were

known. The development of this knowledge occurred during the same period of war and political instability that fostered the establishment of biological warfare programs in the United States⁴³ and elsewhere, and it was evident that the equine encephalitis viruses were optimal candidates for weaponization. The viruses were incorporated into these programs for both potential offensive and defensive reasons. The offensive biological warfare program in the United States was disestablished in 1969 and all stockpiles were destroyed¹³ by executive order, which stated:

The United States shall renounce the use of lethal biological agents and weapons and all other methods of biological warfare. The United States shall confine its biological research to defensive measures such as immunization and safety measures.⁴⁴

Continuing efforts within the US defensive program in the 1960s and 1970s produced four vaccines for the encephalitis viruses: live-attenuated (TC-83) and formalin-inactivated (C84) vaccines for VEEV, and formalin-inactivated vaccines for EEEV and WEEV. These vaccines are used under US Food and Drug Administration IND status for at-risk individuals, distributed under the provisions of the IND, and recommended for use by any laboratory working with these viruses.¹⁰ Although these vaccines are useful, they have certain disadvantages (which are discussed later in this chapter), and next-generation vaccines are being developed.¹⁴

ANTIGENICITY AND EPIDEMIOLOGY

Antigenic and Genetic Relationships

The three American equine encephalitides antigenic complexes, VEE, EEE, and WEE, have been grouped with eight additional virus complexes into the *Alphavirus* genus based on their serologic cross-reactivity (Table 20-1).^{9,45} Analysis of structural gene sequences obtained from members of the VEEV and EEEV complexes confirms the antigenic classification for the most part and serves as another tool for classifying these viruses. Viruses of the WEE complex, including Highlands J, Fort Morgan, and WEEV, have been identified as recombinant viruses originating from ancestral precursors of EEEV and Sindbis virus and fall into a unique genetic grouping of alphaviruses.⁴⁶⁻⁴⁹

Venezuelan Equine Encephalitis Complex

The VEE complex consists of eight closely related viruses that manifest different characteristics with respect to ecology, epidemiology, and virulence for

humans and equids (Table 20-2). The IA/B and IC varieties are commonly referred to as epizootic strains. These strains, which have been responsible for extensive epidemics in North, Central, and South America, are highly pathogenic for humans and equids. All epizootic strains are exotic to the United States and have been isolated only twice since 1973.⁵⁰⁻⁵² Enzootic strains include Everglades (formerly subtype II), Mucambo (formerly subtype IIIA), Pixuna (formerly subtype IV), Cabassou (formerly subtype V), Rio Negro (formerly subtype VI), and varieties ID, IE, and Mosso das Pedras (formerly subtype IF).⁵³⁻⁵⁹ Like the epizootic strains, the enzootic strains may cause disease in humans, but they differ from the epizootic strains in their lack of virulence for equines. Infection of equids with some enzootic subtypes leads to an immune response capable of protecting the animals from challenge with epizootic strains.⁶⁰ Limited data, acquired following laboratory exposures, suggest that cross-protection between epizootic and enzootic strains may be much less pronounced in humans.^{37,61,62}

TABLE 20-1
ANTIGENIC CLASSIFICATION OF ALPHAVIRUSES

Antigenic Complex	Virus		
	Species	Subtype	Variety
Western Equine Encephalitis (WEE)	WEE virus		
	Highlands J virus		
	Fort Morgan virus	Buggy Creek	
	Aura virus		
	Whataroa virus		
	Sindbis virus	Ockelbo Babanki Kyzylgach	
Venezuelan Equine Encephalitis (VEE)	VEE virus	I	A-B
		I	C
		I	D
		I	E
	Mosso das Pedras virus		
	Everglades virus		
	Mucambo virus	Mucambo (IIIA)	
	Tonate virus	Tonate (IIIB) Bijou Bridge (IIIB) 71D-1252 (IIIC)	
	Pixuna virus		
	Cabassou virus		
Eastern Equine Encephalitis (EEE)	Rio Negro virus		
	EEE virus		
	Madariaga virus	Madariaga II	
		Madariaga III Madariaga IV	
Semliki Forest	Semliki Forest virus		
	Bebaru virus		
	Chikungunya virus	Chikungunya	Several
	O'nyong-nyong virus	Igbo Ora	
	Getah virus	Getah	
	Ross River virus	Sagiyama	
	Mayaro virus	Mayaro	
	Una virus		
Middelburg	Middelburg virus		
Nduma	Nduma virus		
Barmah Forest	Barmah Forest virus		
Trocara	Trocara virus		
Southern elephant seal	Southern elephant seal virus		
Eilat	Eilat virus		
Salmon pancreas disease	Salmon pancreas disease virus		1-6 Sleeping disease

Data sources: (1) Nasar F, Palacios G, Gorchakov RV, et al. Eilat virus, a unique alphavirus with host range restricted to insects by RNA replication. *Proc Natl Acad Sci U S A*. 2012;109:14622–14627. (2) King AMQ, Adams MJ, Carstens EB, Lefkowitz EJ, eds. *Virus Taxonomy: Classification and Nomenclature of Viruses: Ninth Report of the International Committee on Taxonomy of Viruses*. San Diego, CA: Elsevier; 2012.

Eastern Equine Encephalitis Virus

The EEEV complex previously consisted of viruses in two antigenically distinct forms: (1)

the North American and Caribbean (NA EEEV), and (2) the South American (SA EEEV). A recent proposal accepted by the International Committee on Taxonomy of Viruses resulted in the reclas-

TABLE 20-2
THE VENEZUELAN EQUINE ENCEPHALOMYELITIS COMPLEX

Subtype	Variety	Prototype Strain	Origin	Cycle	Disease in	
					Horse	Man
VEEV	IA/B	Trinidad donkey	Donkey (Trinidad) ¹	Epizootic	+	+
	IC	P-676	Horse (Venezuela) ²	Epizootic	+	+
	ID	3880	Human (Panama) ³	Enzootic	-	+
	IE	Mena II	Human (Panama) ¹	Enzootic	-	+
Mosso das Pedras virus		78V-3531	Mosquito (Brazil) ⁴	Enzootic	-	?
Everglades virus		Fe3-7c	Mosquito (Florida) ⁵	Enzootic	-	+
Mucambo virus		Mucambo (BeAn8)	Monkey (Brazil) ⁶	Enzootic	-	+
Tonate virus	IIIB	Tonate (CaAn410-D)	Bird (French Guiana) ⁷	Enzootic	-	+
	IIIC	71D-1252	Mosquito (Peru) ⁸	Enzootic	-	?
Pixuna virus		Pixuna (BeAn356445)	Mosquito (Brazil) ⁶	Enzootic	-	?
Cabassou virus		Cabassou	Mosquito (French Guiana) ⁷	Enzootic	-	?
Rio Negro virus		AG80-663	Mosquito (Argentina) ⁹	Enzootic	-	+

Sources that contain original descriptions of or additional information about this strain: (1) Young NA, Johnson KM. Antigenic variants of Venezuelan equine encephalitis virus: Their geographic distribution and epidemiologic significance. *Am J Epidemiol.* 1969;89:286. (2) Walton TE. Virulence properties of Venezuelan equine encephalitis virus serotypes in horses. In: Venezuelan Encephalitis: Proceedings of the Workshop-Symposium on Venezuelan Encephalitis Virus, Washington, DC, 14-17 Sep 1971. Washington, DC: Pan American Health Organization; 1972:134. PAHO Scientific Publication 243. (3) Johnson KM, Shelokov A, Peralta PH, Dammin GJ, Young NA. Recovery of Venezuelan equine encephalomyelitis virus in Panama: a fatal case in man. *Am J Trop Med Hyg.* 1968;17:432-440. (4) Walton TE, Grayson MA. Venezuelan equine encephalitis. In: Monath TP, ed. *The Arboviruses: Epidemiology and Ecology*. Vol 4. Boca Raton, FL: CRC Press; 1988:203-231. (5) Chamberlain RW, Sudia WD, Coleman PH, Work TH. Venezuelan equine encephalitis virus from South Florida. *Science.* 1964;145:272. (6) Shope RE, Causey OR, de Andrade AHP, Theiler M. The Venezuelan equine encephalitis complex of group A arthropod-borne viruses, including Mucambo and Pixuna from the Amazon region of Brazil. *Am J Trop Med Hyg.* 1964;13:723. (7) Karabatsos N. *International Catalogue of Arboviruses Including Certain Other Viruses of Vertebrates*. 3rd ed. San Antonio, TX: American Society for Tropical Medicine and Hygiene; 1985. (8) Scherer WF, Anderson K. Antigenic and biological characteristics of Venezuelan encephalitis virus strains including a possible new subtype isolated from the Amazon region of Peru in 1971. *Am J Epidemiol.* 1975;101:356. (9) Contigiani MS, De Basualdo M, Camara A, et al. Presencia de anticuerpos contra el virus de la encefalitis equina Venezolana subtipo VI en pacientes con enfermedad aguda febril [in Spanish]. *Revista Argentina de Microbiologia.* 1993;25:212-220.

Adapted with permission from Walton TE, Grayson MA. Venezuelan equine encephalitis. In: Monath TP, ed. *The Arboviruses: Epidemiology and Ecology*. Vol 4. Boca Raton, FL: CRC Press; 1989: 206.

sification of the South American variants as a distinct species, Madariaga virus (MADV), within the *Alphavirus* genus.^{63,64} Thus, EEEV will refer to the former NA EEEV, whereas the new species designation, MADV, will refer to the former SA EEEV. The two species can be distinguished readily by hemagglutination-inhibition (HI) assays and plaque-reduction neutralization tests.^{63,65} All EEEV isolates show a high degree of genetic and antigenic homogeneity. However, they are distinct from the MADV isolates, which are more heterogeneous and form three genetic subtypes (II, III, IV).^{66,67} EEEV strains are extremely virulent for humans and horses. Although MADV subtypes infect both horses and humans, infections are rarely associated with significant clinical disease.^{68,69} However, a MADV subtype III virus (ArgM) resulted in up to 75% lethality, depending on the dose, in rodent models infected by aerosol.⁷⁰

Western Equine Encephalitis Complex

Six virus species, including WEE, Sindbis, Aura, Fort Morgan, Highlands J, and Whataroa, comprise the WEEV complex.^{9,64} Several antigenic subtypes of WEEV have been identified, but their geographical distributions overlap.⁵² Most of the members of the WEE complex are distributed throughout the Americas, although Whataroa and subtypes of SINV have strictly Old World distributions.^{9,12} The New World WEE complex viruses can be distinguished readily by neutralization tests. In addition, WEE complex viruses isolated in the western United States (eg, WEEV) are genetically distinct from those commonly found in the eastern United States (eg, Highlands J).^{67,71} SINV is considered a member of the WEEV complex based on antigenic relationships. However, sequence comparisons show that WEEV, Highlands J, and Fort Morgan are actually derived from a

recombination event between ancestral SINV and EEEV (or MADV). The structural domains of the recombinant viruses were derived from the SINV ancestor, whereas the nonstructural domains were derived from the EEEV ancestor.^{67,72}

Epidemiology and Ecology

The evolution of the equine encephalitides is closely tied to the ecology of these viruses in naturally occurring endemic foci. Evidence indicates that the relative genetic homogeneity of the EEE and WEE complex viruses may result from the mixing of virus subpopulations as a result of the movement of the virus from one location to another by the avian hosts.⁷³ In general, these viruses are maintained in a consistently virulent state, capable of initiating epizootics without development of any significant mutations. In contrast, diversity within the VEEV complex results from local evolution of these viruses in mammalian hosts that live in defined habitats. Initiation of epizootic and epidemic activity is almost always associated with appearance of specific genetic change.⁷³

Most commonly, human involvement in the form of endemic and epidemic activity, occurs following intrusion into geographical regions where natural transmission cycles are occurring or following perturbation of these cycles by environmental changes or the addition of other vectors.⁷⁴ The dramatic exception to this is epizootic VEEV, in which the spreading waves of the epizootic among equines can move rapidly over large distances, and humans become infected by mosquitoes that have fed on viremic equines. The high levels of viremia in equines infected with epizootic VEEV make them efficient amplifying hosts, with the result that equine infections normally precede human infections by days to weeks.⁷⁵ Recent evidence suggests that it is the adaptation of these enzootic subtype ID viruses for efficient replication in horses that leads to emergence and efficient epidemic spread of disease.^{19,76} Medical personnel should view with some suspicion evidence of widespread human VEEV infections outside of endemic areas, in the absence of mosquito vectors or in the absence of equine disease, because this combination of circumstances may indicate an unnatural release of virus into the environment.

Enzootic VEEV subtypes, as described above, are maintained efficiently in transmission cycles involving primarily rodents and *Culex* mosquitoes belonging to the subgenus *Melanoconion*.⁷⁷⁻⁷⁹ These mosquitoes live in humid locales with abundant open spaces such as sunny, swampy pastures cut by slowly flowing streams. The mosquitoes are ground feeders,

seldom found higher than 8 meters above ground, and some prefer feeding on mammals rather than birds.⁸⁰ Ground-dwelling rodents, partly because their ecologies are similar to that of the mosquito vectors, are the primary vertebrate hosts for the enzootic forms of VEEV. Following infection, these animals develop viremia of sufficient magnitude and duration to infect mosquitoes feeding on their blood.⁸¹ Other animals, such as bats and certain birds, may play a secondary role.⁸² Seroprevalence rates among human populations living in or near endemic VEEV areas vary but can approach 100%, suggesting that continuous transmission occurs presumably in the absence of significant human disease.⁷⁵ However, virus activity within endemic zones can also be highly focal. In one incident at the Fort Sherman Jungle Operations Training Center in the Panama Canal Zone in December 1967, 7 of 12 US soldiers camped in one area developed VEE disease within 2 days, but another group that camped only a few yards away showed no disease.^{83,84} The incidence of human disease during epizootics also varies, but it is often high. During an outbreak in Venezuela, attack rates of 119 per 1,000 inhabitants per month were reported.⁸⁵ Following an epizootic in Guatemala and El Salvador, overall seroprevalence was estimated at 20%.⁸⁶

Unlike the enzootic strains, the fate of the epizootic strains during interepidemic periods is unclear. The most appealing theory on how epizootic strains arise suggests that they evolve by mutation and equine selection from enzootic strains. Results from oligonucleotide fingerprinting and sequence analysis of ID isolates from Colombia and Venezuela reveal a close similarity to the epizootic strains, suggesting that the equine virulent epizootic strains arise naturally from variants present in populations of ID virus.^{87,88}

Although the genetic evidence indicates that mutation of enzootic strains may lead to the development of epizootic strains, ecological data suggest a strong selective pressure to maintain the enzootic genotype in certain habitats. The enzootic VEEV vector *Culex (Melanoconion) taeniopus* is fully susceptible to both IAB and IE strains following intrathoracic inoculation. Orally exposed mosquitoes are fully competent vectors of the enzootic strain; however, they fail to develop disseminated infection or transmit epizootic virus.^{29,89} In the absence of genetic change, this virus-host interaction appears to be relatively stable. Mosquito resistance to epizootic strains of VEEV is rare. Epizootic strains have been isolated from a large number of mosquito species, and many have been shown to be efficient vectors.⁹⁰ Thus, host switching from enzootic to epizootic vectors may be an important factor in the evolution of epizootic VEEV strains. Researchers suggest that emer-

gence of epizootic strains may result from acquisition of mutations that allow for transmission by abundant, equiphilic mosquitoes. More specifically, adaptation to *Aedes (Ochlerotatus) taeniorhynchus* mosquitoes has been a determinant of some recent emergence events, providing further evidence that the ability to switch hosts is critical for emergence of epizootic strains.⁷⁶ The introduction of mosquito species into previously unoccupied geographical ranges (eg, *Aedes albopictus* into North America) may, therefore, offer the opportunity for epizootic strains to reemerge.

A major outbreak of epizootic VEEV occurred in the late 1960s and early 1970s. Epizootic virus first reached North America in 1969,²⁵ but did not reach the United States until 1971. Studies of this epizootic demonstrated that the virus easily invaded territories in which it was formerly unknown,⁸⁵ presumably as a result of (a) the availability of large numbers of susceptible equine amplifying hosts and (b) the presence of competent mosquito vectors. The initial outbreak in North America, attributed to enzootic strain IE, occurred in 1966 in Tampico, Mexico, involving approximately 1,000 equids.⁹¹

By the end of 1969 and the beginning of 1970, the expansion of the outbreak prompted the Mexican government to request the TC-83 vaccine from the

US Army through the US Department of Agriculture.⁹² Despite the immunization of nearly 1 million equids, the epizootic continued to spread and reached the United States in June 1971. The nature of the virus and the number of human and equine cases prompted the US Secretary of Agriculture to declare a national emergency on July 16, 1971.⁹³ Subsequent immunization of more than 2 million horses and unprecedented mosquito abatement efforts eventually stopped the epizootic before it was able to spread from Texas. Epizootic VEEV has not been isolated in the United States since the 1971 outbreak.

The first large outbreak since the 1969–1971 epizootic occurred in 1995 (Figures 20-1 and 20-2). The epizootic began in northwestern Venezuela and spread across the Guajira peninsula into northeastern Colombia. An estimated 75,000 to 100,000 humans were infected, with more than 20 deaths reported. This outbreak was caused by a VEEV IC strain. By sequence analysis, this strain proved to be essentially identical to a virus that caused an outbreak in Venezuela in 1962–1964.²⁷ Outbreaks of traditionally enzootic strains of VEEV also have



Figure 20-1. This photograph was taken in 1995 near Buena Vista, Colombia. During large Venezuelan equine encephalitis (VEE) epizootics, typical morbidity rates among unvaccinated equines are 40% to 60%, with at least half of the affected animals progressing to lethal encephalitis. Note the disruption of the ground surface, which is caused by the characteristic flailing or swimming syndromes of moribund animals. Although clinically indistinguishable from the syndromes produced by eastern equine encephalitis and western equine encephalitis viruses, the capability of VEE to initiate explosive and rapidly expanding epizootics makes reliable diagnostic tests essential for the initiation of appropriate veterinary and public health measures.



Figure 20-2. This photograph was taken in 1995 near Maicao, Colombia. Equine vaccination is the most effective means available to prevent Venezuelan equine encephalitis (VEE) epizootics as well as to control emerging outbreaks. Equines are the major amplifying hosts, and maintaining a high rate of immunity in the equine population will largely prevent human infection with the epizootic strains of VEE. Both inactivated and live attenuated vaccines are available for veterinary use, but the ability of the live attenuated vaccine to induce immunity in 7 to 10 days with a single inoculation makes it the only practical vaccination strategy in the face of an outbreak. Other measures used to control outbreaks include using insecticides to reduce mosquito populations and prohibiting the transportation of equines from affected areas.

occurred in Mexico and Central America. Genetic analysis confirmed acquisition of mutations and provided further evidence that emergence of epizootic strains may result from the accumulation of genotypic changes in enzootic strains.^{94,95}

EEEV is endemic to focal habitats ranging from southern Canada to Central America. The virus, which has been isolated as far west as Michigan, is most common along the eastern coast of the United States between New England and Florida. Enzootic EEEV transmission occurs almost exclusively between passerine birds (eg, the perching songbirds) and the mosquito *Culiseta melanura*. Because of the strict ornithophilic feeding behavior of this mosquito, human and equine disease requires the involvement of more

general feeders, such as members of the genera *Culex* and *Coquillettidia*. Recent evidence suggests EEEV may overwinter in the southeastern United States in reptiles or amphibians, further necessitating the participation of more general feeding vectors.^{96,97} Mosquito vectors belonging to *Culex* species, subgenus *Melanconion*, may play a role in maintaining and transmitting MADV subtypes.⁹⁸

WEEV is the best studied member of the WEE complex in terms of its epidemiology. The virus is maintained in cycles involving passerine birds and the mosquito *Culex tarsalis*. Humans and equids become infected only tangentially and are considered to be dead-end hosts,⁹⁹ indicating that they do not normally contribute to further spread of the virus in nature.

STRUCTURE AND REPLICATION OF ALPHAVIRUSES

Virion Structure

The alphavirus virion, a spherical particle approximately 65 nm to 70 nm in diameter, is typically composed of three structural proteins enclosing a single molecule of single-stranded RNA. The RNA genome is packaged within an icosahedral nucleocapsid, which is constructed from multiple copies of the capsid (C or CP) protein (Figure 20-3). The nucleocapsid is, in turn, surrounded by a lipid envelope derived from areas of the host cell plasma membrane that had previously been modified by the insertion of two viral glycoproteins. These envelope glycoproteins, E1 and E2, form heterodimers that associate further into trimers^{100,101} to form the short spikes on the surface of the virion. Although a third glycoprotein, E3, was thought to be absent in the mature virion of most alphaviruses, three-dimensional reconstruction of VEEV virions (TC-83 strain) from cryoelectron microscopic images revealed that E3 is associated with the E1–E2 dimers on the virion surface, but at a lower stoichiometry.¹⁰² Evidence of E3 on the surface of Semliki Forest virus virions has also been reported.¹⁰³ However, E1 and E2 dimers—but not E3—are known to be targets of the neutralizing antibody response and are among the determinants of tropism and virulence.^{104,105} Although non-neutralizing, a monoclonal antibody directed against the VEEV E3 protein provided complete protection against an intraperitoneal challenge when administered before exposure.¹⁰⁶ It is possible that this monoclonal antibody protects by binding to the E3 protein at the surface of infected cells blocking the ability of the virus to bud.

Viral Infection

The infection cycle is initiated when the glycoprotein spikes on the virion bind to receptors on the cell

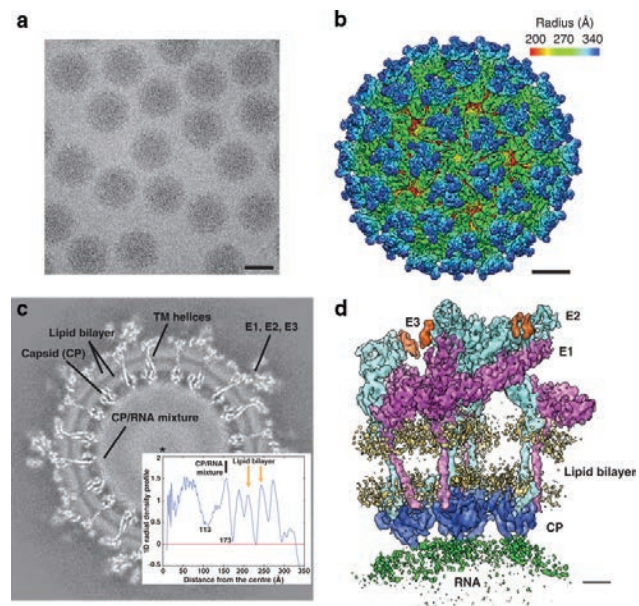


Figure 20-3. 3D structure of Venezuelan equine encephalitis virus (VEEV). (a) A typical CCD image of VEEV TC-83 embedded in vitreous ice. Scale bar: 50nm. (b) Radially colored 3D reconstruction of VEEV, showing the E1 basal triangle (green) and the E2 central protrusion (blue) for each spike. Scale bar: 10nm. (c) A slice through the 3D density map 20 pixels from the origin. The inset is the 1D radial density profile of the map and is aligned to the slice image. (d) One asymmetric unit of the virus containing four unique copies of E1 (magenta), E2 (cyan), E3 (orange), and CP (blue). The cryo-EM densities for the viral membrane (yellow) and genomic RNA (green) are also displayed at a slightly lower isosurface threshold. Scale bar: 2nm.

Data source: Zhang R, Hryc CF, Cong Y, et al. 4.4 Å cryo-EM structure of an enveloped alphavirus Venezuelan equine encephalitis virus. *EMBO J.* 2011;30(18):3854–3863. Reproduced with permission from EMBO.

surface. The virus is localized initially to clathrin-coated pits, where it is engulfed in a coated vesicle and transported to the endosomal compartment within the interior of the cell. A decrease in the pH in the interior of the vesicle induces a conformational change in the glycoprotein spikes, and rearrangement of the E1 glycoprotein mediates fusion of the virion envelope with the endosomal membrane.¹⁰⁷ This fusion results in the release of the nucleocapsid into the cytoplasm, where disassembly of the nucleocapsid releases the viral RNA genome to the synthetic apparatus of the cell.¹⁰⁸

Genomic RNA

The viral genome, a positive-sense RNA of approximately 11,700 nucleotides, has the structural features of messenger RNA (ie, mRNA, a 5' methylated cap [m7GpppA] and a poly-A tract at the 3' end).¹⁰⁹ As a complete and functional mRNA, genomic RNA purified from virions is fully infectious when artificially introduced (ie, transfected) into susceptible cells. Similarly, RNA transcribed from a full-length complementary DNA clone of an alphavirus is also infectious, which allows relatively easy genetic manipulation of these viruses. Mutations introduced into a complementary DNA clone by site-directed mutagenesis are reflected in the RNA transcribed from the altered clone and in the virus produced in transfected cells. These procedures are being used to develop improved vaccines,¹⁴ but they could also be used to enhance specific characteristics required for weaponization.

Structural Protein Synthesis

The alphavirus genome contains two protein coding regions or open reading frames. The 5' 7,500 nucleotides encode a 220,000-dalton precursor polypeptide, which is proteolytically processed to produce the four components of the viral RNA polymerase. The polymerase genes are followed by a second coding region of approximately 3,800 nucleotides, which contains the information that directs the synthesis of the viral structural proteins. Soon after release of the viral genome from the nucleocapsid, the 5' 7,500 nucleotides of the genome RNA are translated to produce the viral RNA polymerase. Early in infection, the incoming viral genome is also used as a template for the synthesis of a negative-sense RNA, identical in length to the genome RNA, but of opposite polarity. The negative-sense RNA subsequently serves as a template for the synthesis of additional genomic RNA. The negative-sense

RNA is also used as a template for transcription of a capped and polyadenylated subgenomic mRNA, which is identical to the 3' third of the genome. The subgenomic mRNA is translated to yield a precursor polypeptide that is proteolytically processed by cotranslational and posttranslational cleavages to produce the viral structural proteins. The order of the structural proteins within the precursor is C-E3-E2-6K/TF-E1.

As the subgenomic mRNA is translated, the C protein is produced first and catalyzes its own cleavage from the nascent polypeptide soon after the ribosome transits into the sequences that encode E3. After release of the C protein, the free amino terminus of E3 is bound to the membranes of the rough endoplasmic reticulum. As the synthesis of nascent E3 and E2 (precursor E2 or pE2) continues, the polypeptide is translocated into the lumen of the endoplasmic reticulum, where oligosaccharides and fatty acids are added.¹¹⁰ A domain of hydrophobic amino acids near the carboxyl terminus of E2 inhibits further transmembranal movement so that the last 30 to 40 amino acids of the E2 polypeptide remain exposed on the cytoplasmic side of the membrane. The 6K polypeptide serves as a signal for membrane insertion of the second glycoprotein, E1, and is subsequently cleaved from both E2 and E1 by signal peptidase.¹¹¹ A hydrophobic anchor sequence present near the carboxyl terminus of E1 secures the protein in the membrane.

Budding and Release of Progeny Virus Particles

Soon after synthesis, the precursor of PE2 and E1 interact to form multimeric complexes,¹¹² which are then transported through the Golgi apparatus, where the final modifications of the oligosaccharide are made. The pE2 protein is cleaved to generate the mature E2 and E3 glycoproteins soon after the glycoproteins leave the Golgi apparatus,¹¹³ and the mature viral spikes assume an orientation in the plasma membrane with the bulk of the E2 and E1 polypeptides exposed on the exterior surface of the cell. In vertebrate cells, final assembly of progeny virus particles happens by budding exclusively at the plasma membrane¹¹⁴; whereas in cultured arthropod cells, budding also occurs at intracellular membranes.¹¹⁵

In vertebrate cells, budding is initiated when intracellular nucleocapsids bind to the 30 to 40 amino acid cytoplasmic domain of the E2 glycoprotein,¹¹⁶⁻¹¹⁸ inducing the formation of a locally ordered array of glycoprotein spikes, which excludes most of the cellular membrane proteins from the region.

Additional lateral associations between the individual spikes stabilize the lattice and promote additional E2-C protein interactions. The growing lattice may draw the membrane around the nucleocapsid, completing the envelopment with the release of the spherical virus particle. Maximal amounts of virus are typically produced from mammalian cells within 8 to 10 hours after infection, and disintegration of the infected cell is likely caused by programmed cell death

(apoptosis) rather than direct effects of the virus on cellular function.¹¹⁹ In contrast, alphaviruses initially replicate to high titer in arthropod cells with little or no evidence of cytopathology. The surviving cells continue to produce lesser amounts of virus, often for weeks or months. The ability of the virus to replicate without causing cell death in arthropod cells may be critical for maintenance of the virus in the mosquito vector in nature.

PATHOGENESIS

In humans, the pathogenesis of VEEV, EEEV, and WEEV infections acquired by aerosol, which is the route of greatest biological defense concern, is unknown. Little is known of the pathogenesis following natural vectorborne infections of humans, mainly because of the limited autopsy material. Much of the information on VEEV pathogenesis in humans is based on a histological review of 21 human fatalities from the 1962–1963 VEEV epidemic in Zulia, Venezuela.¹²⁰ With few exceptions, the histopathological lesions in these cases, all among children or young adults, were comparable to those observed in experimentally infected animals. Tissues commonly affected in both humans and animals^{121–129} include those of the lymphoid and reticuloendothelial systems as well as the central nervous system (CNS). Widespread hepatocellular degeneration and interstitial pneumonia, not ordinarily seen in experimental animals, were frequent histological findings in these cases of severe human disease. Much of the understanding of the pathogenesis of VEEV, EEEV, and WEEV has relied on animal studies.

The clinical and pathological responses of the host to VEEV, EEEV, and WEEV infection are highly dependent on a number of host and viral factors, including:

- the species, immune status, and age of the host;
- the route of infection; and
- the strain and dose of virus.

Most of the existing experimental data are from studies using rodent models challenged with the virulent Trinidad donkey (TrD) strain of VEEV, an epizootic IAB serotype virus, or its genetic clone V3000. A few NHP studies have also been reported.^{130,131} In animal models, as in humans, the lymphatic system and the CNS are consistent target organs. However, the relative degree of injury caused to these tissues varies. Virulent VEEV causes limited and reversible lesions to the lymphoid organs of mice and NHPs,^{122,126} but in guinea pigs and hamsters, it causes extreme and irreversible damage to those organs.^{127,128} As a

result, in the guinea pig and hamster models, death occurs before serious CNS disease develops.^{124,125} The host species and the route of administration of VEEV greatly affect CNS disease development. Mice uniformly exhibit a severe paralytic episode before death from diffuse encephalomyelitis following peripheral or aerosol administration of TrD or V3000.^{122,126,132,133} NHPs, however, exhibit few if any clinical signs of encephalitis following peripheral inoculation with TrD, and only modest perivascular cuffing and gliosis, mainly in the thalamus, hypothalamus, and olfactory areas of the brain.^{122,123} In one study, NHPs inoculated by the intraperitoneal route developed transient viremia and biphasic fever but otherwise exhibited no evidence of clinical disease.¹²² NHPs in this study developed brain lesions as early as 6 days postinfection, which typically included lymphocytic perivascular cuffs and gliosis, with the thalamus being the site of the most intense inflammation.¹²² NHPs infected by intranasal inoculation had more moderate inflammation, especially in the cortex and hypothalamus,¹³⁴ whereas a Colombian epizootic strain of VEEV given by aerosol caused severe clinical and pathological CNS signs and resulted in death in approximately 35% of rhesus macaques.¹²³ In another study, cynomolgus macaques infected with the VEEV IE or Mucambo virus (IIIA) developed fever, viremia, and mild clinical signs of encephalitis (tremors, loss of coordination) but recovered.¹³⁵ Both mice and cynomolgus macaques challenged intracerebrally with TrD or related VEEV strains developed severe and lethal neurological signs with moderate to severe brain histopathology.^{134,136}

The mechanisms of neuroinvasion by VEEV represent an important issue, particularly regarding immunoprophylaxis. The specific mechanism of neuroinvasion in the case of peripheral inoculation of virus is not completely understood; however, important features of the process have been elucidated by animal studies. In mice inoculated peripherally and subsequent to the development of viremia, virulent VEEV is detectable in the brain, initially in the olfactory bulbs, and usually within 48 hours of infection.^{133,137,138}

It appears that virus in the blood escapes from fenestrated capillaries supplying the olfactory lining of the nasal tract. Virus may then invade olfactory neuron cell bodies or their axons and may be carried via the olfactory nerves into the olfactory bulbs of the brain. However, surgical or chemical ablation of the olfactory lining did not significantly affect the mortality rate or average survival time of infected mice. In this case, neuroinvasion was suspected to occur via the trigeminal nerves.¹³⁷ However, direct invasion of the brain across the blood–brain barrier^{125,139} seems less compelling than the olfactory route.

The understanding of the mechanism of neuroinvasion following respiratory infection is more clear. In mice, an early and strong target of virulent VEEV administered by aerosol has been shown to be the olfactory neuron.¹³³ This cell type, a so-called “bipolar neuron,” is in direct contact with inspired air at one pole and synapses with resident neurons in the olfactory bulb at the opposite pole, offering a direct connection to the brain independent of viremia development. Both the nasal olfactory epithelium and the olfactory nerve axon bundles in the underlying connective tissue exhibit VEEV antigen within 24 hours of aerosol infection (Figure 20-4), and the olfactory bulbs also show viral infection shortly thereafter (Figure 20-5). In a study of rhesus macaques inoculated intranasally with VEEV, the virus gains access to the olfactory bulb within 24 hours after infection and before the onset of viremia, suggesting direct neuroinvasion via olfactory neurons similar to neuroinvasion in the mouse.¹⁴⁰

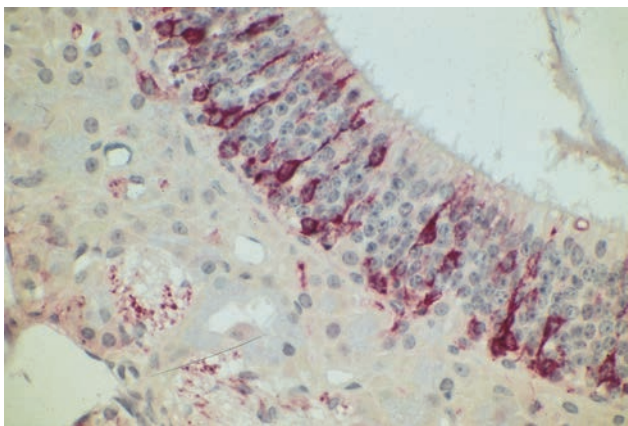


Figure 20-4. Nasal tissue, BALB/c mouse, 2 days after exposure to aerosolized Venezuelan equine encephalitis (VEE) virus. Note immunoreactive olfactory epithelium and olfactory nerves. Alkaline phosphatase-labeled streptavidin method using rabbit antiserum to VEE virus (Mayer’s hematoxylin counterstain, original magnification $\times 300$).

However, in inoculated macaques whose olfactory nerves had been surgically removed, VEEV was still able to reach the olfactory bulb by 36 hours after infection, presumably by the vascular route. Although the olfactory bulb and olfactory tract were sites of early viral replication, the virus did not appear to spread to the rest of the brain along the neural tracts in these monkeys, as it does in mice. In a more recent study in which cynomolgus macaques were exposed to VEEV by the aerosol route, virus was not detected in the brain until 4 days postinfection and was only detected in the region of the olfactory tubercle.^{132,141} However, the dose delivered to the cynomolgus macaques in this study was not reported, which could influence the resulting pathology. The teeth are another early target of VEEV administered peripherally or by aerosol,^{132,133,137} and the trigeminal nerves appear to carry VEEV from the teeth into the brains as an alternate, although it is probably a less significant route of neuroinvasion.

The pathogenesis of EEEV has not been as thoroughly evaluated. In contrast to VEEV, EEEV replication in lymphoid tissues is limited by tissue-specific microRNA¹⁴²; primary EEEV replication after subcutaneous inoculation occurs in fibroblasts, skeletal muscle, and osteoblasts.¹³³ In a recent study, it was shown that when mice are infected with EEEV strain FL93-939 by either the aerosol or intranasal route that the virus specifically targets the olfactory epithelium and enters the brain via the olfactory tract.^{143,144} In mice exposed by the aerosol route, virus was detected in the brain as early as 6 hours postinfection. In mice inoculated by the subcutaneous route, the mechanism of neuroinvasion

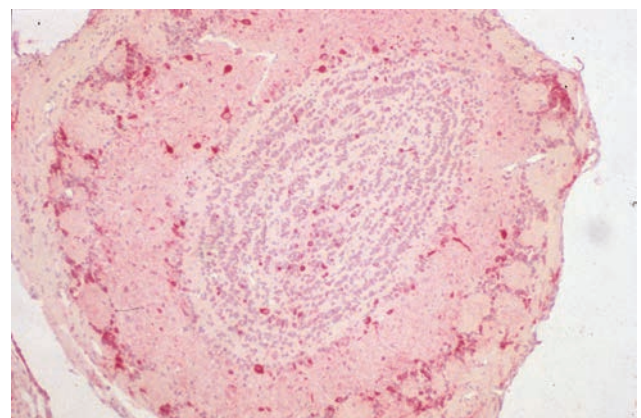


Figure 20-5. Olfactory bulb, BALB/c mouse, 2 days after exposure to aerosolized Venezuelan equine encephalitis (VEE) virus. Note immunoreactive cells. Alkaline phosphatase-labeled streptavidin method using rabbit antiserum to VEE virus (Mayer’s hematoxylin counterstain, original magnification $\times 150$).

is less clear and the virus may enter the brain either by the olfactory tract or the vascular route. In all cases, once in the brain, the neuron is the main viral target and animals exhibited varying degrees of neuronal cell death and meningoencephalitis.^{143,144}

In a study in which guinea pigs were infected with EEEV by aerosol, animals developed clinical signs within 24 hours of infection and rapidly progressed to include circling, recumbency, coma, and death.⁷¹ No difference in virulence or time to death was seen whether virus was targeted to the lower respiratory tract or upper respiratory tract by manipulating particle size. In these animals, virus was found in the olfactory epithelium and olfactory bulbs followed by spread to all parts of the brain by 4 days postinfection. Again, the neuron was the main viral target and brain lesions included neuronal necrosis, perivascular cuffs, and encephalitis with vasculitis noted in few animals in late-stage cases.⁷¹

EEEV natural history and pathogenesis studies in NHPs are limited. However, in a recent natural history study cynomolgus macaques were challenged with aerosolized EEEV. In this study, 66% of the animals exhibited fever, leukocytosis, and neurological signs and succumbed 5 to 9 days postchallenge.¹⁴⁵ The major pathological changes in the brain included severe meningoencephalomyelitis characterized by neuronal necrosis, perivascular cuffs, gliosis, satellitosis, edema, hemorrhage, and vasculitis in some animals.¹⁴¹

Since there has been a dramatic decline in the incidence of WEEV infection in humans and horses since the middle of the 20th century, few animal studies with this virus have been conducted in recent years. In one study, mice were infected with several strains of WEEV

by various routes.¹⁴⁶ The McMillian strain of WEEV was 100% lethal by the intracranial, subcutaneous, and aerosol routes and 90% lethal by the intravenous and intranasal routes. Histopathological lesions occurred in the brains of all mice and were characterized by neuronal necrosis, edema, lymphocytic meningitis, and occasional fibrin thrombi. Two studies in hamsters using various strains of WEEV^{147,148} showed lethality by various routes of infection with the major histopathological lesions in the brain being neuronal necrosis, lymphocytic meningitis and perivascular cuffs, astrocytosis, microgliosis, and hemorrhage. In a recent study, cynomolgus macaques were infected with the CBA-87 strain of WEEV by aerosol.¹⁴⁹ Affected animals developed fever, inappetence, lethargy, and tremors, as well as leukocytosis and hyperglycemia. The histopathological lesions were characterized as nonsuppurative meningoencephalomyelitis. In particular, infection was noted in Purkinje cells in the cerebellum and hypothalamus, the region of the brain that controls body temperature.

The specific viral and host mechanisms that contribute to neuroinvasion and neurovirulence for each of these viruses have yet to be elucidated. The importance of these mechanisms and the differences observed between peripheral and aerosol administration are of significant practical concern because the immunological mechanisms of virus neutralization respective to each route can vary greatly, as studies have shown.^{130,150,151} The efficiency and rapidity of neuroinvasion following aerosol infection also place very high demands on the vaccines used for immunoprophylaxis (vaccines are discussed later in this chapter).

CLINICAL DISEASE AND DIAGNOSIS

VEEV, EEEV, and WEEV are also recognized for their potential for neuroinvasion and encephalitis in humans, sometimes in epidemic proportions. However, many of the infections caused by these viruses are manifested as systemic viral febrile syndromes, and infections by EEEV and WEEV may remain subclinical. Furthermore, these alphaviruses vary markedly in both their neurotropism and the severity of their neurological sequelae. Depending on the virus, patients presenting with the general syndrome of alphavirus encephalitis have a varying combination of fever, headache, confusion, dysphasia, seizures, paresis, ataxia, myoclonus, and cranial nerve palsies.

Venezuelan Equine Encephalitis

The IAB and IC subtypes of VEEV, which are pathogenic for equines, have the capacity for explosive epizootics with epidemic human disease.

Epidemics of VEE affecting 20,000 to 75,000 people have been documented in Colombia, Venezuela, and Ecuador.^{25,75,152} In contrast to the other alphavirus encephalitides (EEEV and WEEV), epizootic strains of VEEV are mainly amplified in equids, rather than birds, so that equine disease normally occurs before reports of human disease. Enzootic VEEV strains (subtypes ID and IE, as well as Mosso das Pedras, Everglades, Mucambo, Tonate, Pixuna, Cabassou, and Rio Negro viruses, previously known as VEEV subtypes IF, II, IIIA, IIIB, IV, V, and VI, respectively) are not recognized as virulent for equines, but disease has been documented with most of these variants in humans who reside in or move into enzootic foci, or after laboratory infections (see Table 20-2). The resulting syndromes appear to be similar—if not indistinguishable—from the syndrome produced by epizootic variants, which ranges from undifferentiated febrile illness to fatal encephalitis. In NHPs,

aerosol exposure to enzootic strains results in a febrile illness with indications of encephalitis virtually indistinguishable from that seen with epizootic strains in terms of onset, severity, and duration.¹⁵³

Following an incubation period that can be as short as 28 hours but is usually 2 to 6 days, patients typically develop a prostrating syndrome of chills, high fever (38°C–40.5°C), headache, and malaise.¹⁵⁴ Photophobia, sore throat, myalgias, and vomiting are also common symptoms. Frequent signs noted on physical examination include conjunctival injection, erythematous pharynx, and muscle tenderness. Although essentially all human infections with VEEV are symptomatic,^{83,84} only a small percentage manifest neurological involvement.¹⁵⁵ In one epidemic, the ratio of encephalitis to infections was estimated at less than 0.5% in adults, although possibly as high as 4% in children.¹⁵⁶ Mild CNS involvement is evidenced by lethargy, somnolence, or mild confusion, with or without nuchal rigidity.⁸ Seizures, ataxia, paralysis, or coma indicate more severe CNS involvement. In children with overt encephalitis, case fatalities may be as high as 35% compared with 10% for adults.¹⁵⁷ However, for those adults who survive encephalitic involvement, neurological recovery is usually complete,¹⁵⁸ although one report documented motor disorders and an increased incidence of seizures in children following VEE outbreaks.¹⁵⁸ Abortions and increased fetal deaths have also been attributed to VEEV infection.^{27,152} School-aged children are believed to be more susceptible to a fulminant form of disease, which follows a lethal course over 48 to 72 hours in which depletion of lymphoid tissues is prominent.^{120,159,160}

In the first 3 days of illness, leukopenia and elevated aspartate aminotransaminase are common. For those with CNS involvement, a lymphocytic pleocytosis of up to 500 cells per microliter can be observed in the cerebrospinal fluid (CSF). The CSF pleocytosis may be acutely polymorphonuclear but soon becomes predominantly lymphocytic.

Specific diagnosis of VEEV infection can be accomplished by virus isolation, serologic testing, identification of virus-specific nucleic acid, or all three.^{59,161–165} Identification of the VEEV subtype of an isolate involved can usually be determined by cross-neutralization tests or more reliably by genetic sequence analysis. During the first to third days of symptoms of nonspecific febrile illness, VEEV may be recovered from either the serum or the nasopharynx.¹⁶⁶ Despite the theoretical possibility of person-to-person transmission of virus present in the nasopharynx, no such occurrences have been reported. In NHPs, the virus is found in the blood for the first 2 to 3 days after aerosol exposure but levels are low compared to what has been reported for natural infection and may not be

detectable after fever onset for enzootic strains.^{131,153} VEEV can be isolated from the nasopharynx of NHPs for up to 5 days postexposure after aerosol exposure of naïve animals. Antibodies detectable by an HI assay, enzyme-linked immunosorbent assay, or plaque-reduction neutralization tests appear as viremia diminishes. Complement-fixing antibodies make their appearance later during convalescence. VEEV immunoglobulin M (IgM) antibodies are present in acute phase sera,⁸⁴ and VEEV IgM tests do not react with sera from patients with EEEV or WEEV.¹⁶⁷ Since patients with encephalitis typically come to evaluation later in the course of clinical illness, virus is recovered less often from them,¹³¹ and they usually have virus-specific serum antibody by the time of clinical presentation.¹⁶⁸ Immunity after infection is probably lifelong to the homologous serotype, but cross-immunity may be weak or nonexistent to heterologous serotypes.^{37,61,62} Thus, when viewed either as an endemic disease threat or as a potential biological warfare threat, adequate immunization may require polyvalent vaccines.

Eastern Equine Encephalitis

In North America, EEEV is maintained in a natural transmission cycle between *Culiseta melanura* mosquitoes and passerine birds in freshwater hardwood swamps and forested areas, primarily in the Atlantic and Gulf Coast states. EEEV outbreaks are typically recognized when severe equine or human encephalitis occurs near such areas.¹⁶⁹ In the southeastern United States, *Culex erraticus* may play an important role in the transmission of the virus to humans and horses.^{170–172} During vectorborne EEEV epidemics, the incidence of human infection is low (<3% of the population at risk), and the neurological attack rate in one outbreak was estimated at 1 in every 23 cases of human infection.¹⁷³ However, the effect on morbidity and mortality of aerosol-acquired (the expected route of infection in a biological warfare offensive) EEEV infection in humans is unknown, although animal studies suggest that EEEV by aerosol is lethal.¹⁴⁵ The incubation period in humans varies from 5 to 15 days. Adults typically exhibit a febrile prodrome for up to 11 days before the onset of neurological disease.¹⁷⁴ However, illness in children exhibits a more sudden onset.¹⁷⁵ In natural outbreaks, viremia occurs during the febrile prodrome,¹⁷⁶ but is usually undetectable by the time clinical encephalitis develops, when HI and neutralizing antibodies become evident.¹⁷³ Despite the development of a prompt and neutralizing humoral response, the virus is not eliminated from the CNS, and progressive neuronal destruction and inflammation continue.

EEE is the most severe of the arboviral encephaliti-
des, with high mortality and severe neurological se-
quelae.¹⁷⁷ During EEEV outbreaks, the attack, morbidity,
and fatality rates are highest in young children¹⁷⁸
and elderly people.¹⁷⁹ Overall, approximately 4% to 5%
of human EEEV infections result in clinically apparent
EEE in the United States, with an average of six human
cases of EEE reported annually.¹⁸⁰ Case fatality rates
are estimated to be from 30% to 70%, but asymptomatic
infections and milder clinical illness are under-
reported. The illness is characterized by a nonspecific
prodrome followed by severe headache, high fevers,
lethargy, and seizures, often with rapid progression
to coma and death.^{181–183} Motor involvement with pa-
resis is common during the acute phase of the illness.
Major disturbances of autonomic function, such as
impaired respiratory regulation or excess salivation,
may dominate the clinical picture. Between 30% and
70% of survivors are left with long-term neurologi-
cal sequelae such as seizures, spastic paralysis, and
cranial neuropathies. Cognitive impairment ranges
from minimal brain dysfunction to severe dementia.

In a recent retrospective study of 15 cases of EEE in
children, fever, headache, and seizures were the most
common clinical signs, with 87% of the patients becoming
stuporous or comatose during the first 3 days of hospital-
ization.¹⁸⁴ Radiographic lesions were noted in the basal
ganglia, thalami, and cerebral cortex. This study found
an important association between a short prodrome
(ie, the time between initial nonspecific symptoms and
the first major neurologic symptom) and an increased
risk for death or for severe disease. The eight patients
who had a poor outcome all had a prodrome of 2 days
or less, and all four deaths occurred in this group.¹⁸⁴

Clinical laboratory findings in patients with EEE
often demonstrate an early leukopenia followed by a
leukocytosis. Elevated opening pressure is commonly
noted on lumbar puncture and, especially in children,
the CSF lymphocytic pleocytosis may reach a cell
count of thousands of mononuclear cells per micro-
liter. However, in a recent report, neutrophilic pleo-
cytosis with elevated levels of protein were the most
consistent findings when CSF was evaluated within
the first week of symptom onset.¹⁸⁴ Specific diagnosis
of EEE depends on virus isolation or serologic testing
in which rising titers of HI, complement-fixing, or
neutralizing antibodies are observed. IgM antibod-
ies are usually detectable in acute-phase sera.¹⁶⁷ As
with other alphaviruses, neutralization tests are the
most specific. Immunohistochemistry can also be
performed postmortem on fixed brain samples.¹⁴⁵ In
NHPs exposed by aerosol to EEEV, the period from
fever onset until the animal is moribund is less than
48 hours regardless of dose.¹⁴⁵

Western Equine Encephalitis

Like VEEV, naturally acquired WEEV (by mosquito
bite) is less virulent for adult humans than for equids
and children, with lower rates of fatalities and neuro-
logical sequelae.¹⁸⁵ As with EEEV, infants and elderly
people are especially susceptible to severe clinical illness
and neurological sequelae, with case fatality rates of
about 10%. Highlands J virus, an antigenically related
member of the WEE complex that is isolated frequently
in the eastern United States, rarely infects humans.

The incubation period is 5 to 10 days for natural
WEEV infection. In NHPs infected by aerosol, the
incubation period is 4 to 5 days.¹⁴⁹ A large percent-
age of patients with vectorborne infections are either
asymptomatic or present with a nonspecific febrile
illness or aseptic meningitis. The ratio of encephalitis
cases per infection has been estimated to vary from 1
per 1,150 in adults, to 1 per 58 in children, to 1 per 1
in infants.⁷⁴ However, the severity of the syndrome
and the incidence of inapparent infection almost
certainly depend on the strain and dose of the virus,
and the route of infection. Some unusual isolates
show high virulence in laboratory animals^{138,186,187}
and in one study of laboratory-acquired infections
in adults, two of five patients died.³⁵ Symptoms
usually begin with malaise, headache, and fever,
followed by nausea and vomiting.¹⁸⁸ Telemetry data
from NHPs exposed to WEEV by aerosol revealed, in
addition to fever, increases in heart rate and changes
in electrocardiograph readings, indicative of sinus
tachycardia.¹⁸⁹ A transient leukopenia followed by a
pronounced leukocytosis composed almost entirely
of segmented neutrophils correlated with a poor
prognosis. Fever severity also correlated with a poor
prognosis. Over the next few days the symptoms in-
tensified, and in some cases, somnolence or delirium
progressed into coma. The severity of neurological
involvement is inversely related to age, with more
than 90% of children younger than 1 year exhibiting
focal or generalized seizures.¹⁹⁰ Physical examina-
tion typically reveals nuchal rigidity, impaired
sensorium, and upper motor neuron deficits with
pathologically abnormal reflexes.

Patients with the most severe disease usually die
within the first week of clinical illness, with case
fatalities averaging 10%. Other patients begin a grad-
ual convalescence after the first week of encephalitic
symptoms. Most adults recover completely, but it may
take months to years to recuperate from fatigability,
recurrent headaches, emotional lability, and impaired
concentration.¹⁹¹ Some patients have permanent
residua of motor weakness, cognitive deficits, or a
seizure disorder. Children carry a higher incidence of

neurological sequelae, ranging from less than 1% in those older than 1 year, to 10% in infants 2 to 3 months old, to more than 50% in newborns. Congenital infection in the last trimester of pregnancy has been described, with resultant encephalitis in the infants.¹⁹² Laboratory accidents involving aerosol exposure to WEEV have been documented and the mortality of those limited cases was 40%.³⁵ In NHPs, aerosol exposure to a dose equivalent to 10 times the median infective dose produced fever, and 50% of the animals developed clinical signs indicative of encephalitis. Twenty-five percent of those animals died from the infection by day 9 postexposure.¹⁸⁹

Viremia is rarely detectable by the time patients present with encephalitic symptoms, but IgM, HI, and neutralizing antibodies are generally detected by the end of the first week of illness, and they increase in titer during the following week.^{167,193,194} In NHPs exposed to aerosolized WEEV, the virus was not detectable in the serum or nasopharynx postexposure.¹⁸⁹ However, low levels of virus were detected in CSF. Antibody responses were not detectable by an enzyme-linked immunosorbent assay or plaque-reduction neutralization test until day 9 postexposure in survivors, which was after control animals had died from the infection. Complement-fixing serologic responses generally appear in the second week and rise thereafter. Isolation of virus with up to 4-fold increase in titer is diagnostic, but because of serologic cross-reactions with other alphaviruses, neutralization tests are preferred. Examination of the CSF reveals a lymphocytic pleocytosis ranging from 10 to 400 mononuclear cells per microliter. WEEV may occasionally be isolated from the CSF taken within the first 2 days of fever, and it is frequently recovered from brain tissue on postmortem examination.¹⁹⁵ Survival from natural infection presumably confers long-term immunity; however, it may not protect against aerosol exposure.¹⁹⁶

Differential Diagnosis of Alphavirus Encephalitis

Most acute infections with VEEV and WEEV produce a moderately severe but nonspecific clinical illness, consisting of fever, headache, and myalgias. Therefore, in a potential biological warfare scenario, alphaviruses should be considered in the differential diagnosis whenever epidemic febrile illness occurs, especially if several patients progress to neurological disease. Sick or dying equids near an epidemic febrile illness among troops should immediately suggest the possibility of large-scale alphavirus exposure. Other potential biowarfare agents that may infrequently produce or imitate a meningoencephalitic syndrome

include Rift Valley fever virus, *Brucella* species, *Yersinia pestis*, *Salmonella typhi*, *Coxiella burnetii*, and botulinum toxin. As with any meningoencephalitis diagnosis, it is imperative to rule out any potential cause that may be specifically treatable.

For encephalitis cases that are more sporadic in their occurrence, other important viral etiologies that might not be readily discriminated from the alphaviruses by

TABLE 20-3
SOME IMPORTANT VIRAL CAUSES* OF ENDEMIC ENCEPHALOMYELITIS

Virus Family	Genus	Species
<i>Togaviridae</i>	<i>Alphavirus</i>	Eastern equine encephalitis virus Western equine encephalitis virus Venezuelan equine encephalitis virus
<i>Flaviviridae</i>		St Louis encephalitis virus Murray Valley encephalitis virus West Nile virus Japanese encephalitis virus Dengue virus Tickborne complex viruses
<i>Bunyaviridae</i>		LaCrosse virus Rift Valley fever virus Toscana virus
<i>Paramyxoviridae</i>	<i>Paramyxovirus</i> <i>Morbillivirus</i> <i>Henipavirus</i>	Mumps virus Measles virus Hendra virus Nipah virus
<i>Arenaviridae</i>	<i>Arenavirus</i>	Lymphocytic choriomeningitis virus Machupo virus Junin virus Guanarito virus
<i>Picornaviridae</i>	<i>Enterovirus</i>	Poliovirus Coxsackievirus Echovirus
<i>Reoviridae</i>		Colorado tick fever virus
<i>Rhabdoviridae</i>	<i>Lyssavirus</i>	Australian bat lyssavirus Rabies virus
<i>Herpesviridae</i>	<i>Herpesvirus</i>	Herpes simplex virus types 1 and 2 Epstein-Barr virus Cytomegalovirus
<i>Adenoviridae</i>	<i>Adenovirus</i>	

*Not all-inclusive.

EXHIBIT 20-1

NONVIRAL CAUSES OF ENCEPHALOMYELITIS

Treatable infectious conditions that can mimic viral encephalitis:

- Partially treated bacterial meningitis
- Brain abscess
- Subdural empyema
- Embolic encephalitis associated with bacterial endocarditis
- Lyme disease
- Tuberculous meningitis
- Fungal meningitis
- Rocky Mountain spotted fever
- Cat scratch disease
- Cerebral malaria
- Trypanosomiasis
- Toxoplasmosis

Vascular, autoimmune, and neoplastic diseases that can mimic infectious meningoencephalitis:

- Lupus cerebritis
- Cerebral and granulomatous arteritis
- Lymphomatous cerebritis
- Whipple's disease
- Behçet syndrome
- Carcinomatous meningitis

clinical features are listed in Table 20-3. This list is not all-inclusive but suggests other viral encephalitides that should be considered if a patient presents, a priori, with an encephalitic syndrome.

Epidemiological, historical, and laboratory information remains critical to differential diagnosis. Immediate and careful consideration must be given to treatable infections that may mimic viral encephalitis (Exhibit 20-1) because prompt and appropriate intervention can be lifesaving. In addition, vascular, autoimmune, and neoplastic diseases may imitate infectious meningoencephalitis.

For endemic meningoencephalitic disease that occurs outside biowarfare theaters, the geographical locale and the patient's travel history are of preeminent

importance in diagnosing an arboviral encephalitis. Risk for disease is increased relative to the patient's amount of arthropod contact near swampy or forested areas during the summer in temperate climates or year-round in the tropics. Encephalitic illness of equids in the surrounding locale is an important indication of ongoing transmission of encephalitic alphaviruses. Animal studies have indicated that the virus may not be detectable in the serum during the febrile period, and antibody responses may be weak or nonexistent, making diagnosis difficult, which is particularly true for WEEV. Examination of the CSF, including viral cultures or reverse transcription polymerase chain reaction, is critical in differentiating bacterial from viral infections, and infectious from noninfectious etiologies. Serum and CSF tests based on antibody or genetic detection hold great promise in more rapid diagnosis of infectious encephalitis. In some instances it will be necessary to (a) institute therapy for possible, treatable, infecting organisms and (b) await definitive laboratory diagnostic tests.

Medical Management and Prevention

No licensed vaccines or therapeutics currently exist for the alphaviral encephalitides; therefore, treatment is aimed at management of specific symptoms (eg, anticonvulsant medication and airway protection). The high fever occasionally produced by WEEV infection in humans is a special problem among the arboviral encephalitides that may require aggressive antihyperthermia measures.^{194,197} The US Army has extensive experience with IND live-attenuated and formalin-inactivated vaccines in humans (which are discussed later in this chapter).

Use of an effective vaccine in horses would also prevent outbreaks of epizootic VEE, as equines are the major amplifying species for VEEV. However, vaccination of horses is not a useful public health tool for EEEV, WEEV, or enzootic VEEV, because horses are not important as amplifying hosts for these viruses. Integrated mosquito control measures can also have significant impact on ameliorating epidemic transmission.

IMMUNOPROPHYLAXIS

Relevant Immune Effector Mechanisms

The equine encephalitis viruses constitute both an endemic disease threat as well as a biological warfare threat; therefore, adequate immunoprophylaxis of military and civilian personnel will require protection against both vectorborne and aerosol-acquired

infections. The requirements for protection against parenteral infection are well described, but the requirements for protection against infectious aerosols are more stringent and remain largely unidentified. Within a few days of infection with an alphavirus, specific antibodies can be detected in the serum of animals or humans. Within 7 to 14 days, a virus-neutralizing

antibody response develops, as measured by the ability of serum antibodies to block virus infectivity *in vitro* or *in vivo*. Protection from mosquito-vectored alphavirus disease is believed to be primarily mediated by this virus-specific neutralizing antibody response, which is largely directed against epitopes on the E2 glycoprotein.^{198,199} Protection mediated by nonneutralizing antibodies to alphaviruses, directed largely at epitopes on the E1 glycoprotein, has also been described.^{196,200} Protection from aerosol exposure correlated with serum neutralization or antibody titers in some studies in mice, hamsters, and NHPs,^{130,131,135,198,201} but this is not consistently the case.

There have also been reports of virus-specific cytotoxic T cell responses induced against alphaviruses.²⁰²⁻²⁰⁴ Although cytotoxic T cell activity was not detected in early studies with a VEEV vaccine in mice,²⁰⁵ more recent studies have demonstrated a role for certain subsets of T cells in protection against VEEV.²⁰⁶⁻²⁰⁸

Nonspecific immune responses that occur following alphavirus infection include the induction of secretion of interferon (IFN)²⁰⁹⁻²¹³ and the activation of cytotoxic macrophages.²¹⁴ Several studies have demonstrated the importance of the innate immune response, specifically IFN- α , in resistance to alphavirus infection. Studies with Semliki Forest virus and VEEV have shown that IFN α/β receptor knockout mice are more susceptible to infection.²¹⁵⁻²¹⁷ Pre- and postexposure administration of IFN or inducers of interferon *in vivo* may be effective for protection against alphaviruses.^{218,219} IFN- β was beneficial in protection against the Semliki Forest virus peripheral challenge when administered up to 6 days postexposure. Mice were resistant to subcutaneous challenge with VEEV TrD and partially protected from inhalation challenge when administered pegylated IFN- α on days -2 and +5 relative to exposure.²²⁰ Pretreating mice with polyinosinic:polycytidylic acid (poly I:C) afforded partial protection against peripheral challenge with EEEV,²¹⁵ and poly I:C with added carboxymethylcellulose and poly-L-lysine similarly induces protection against respiratory challenge with WEEV.²¹⁹ Although these studies clearly indicate the importance of interferons in host resistance to alphavirus infections, further study is necessary to determine the efficacy of IFN- α for prophylactic or therapeutic use in humans.

Passive Immunization

Passive transfer of neutralizing antisera or monoclonal antibodies to naive recipients protects animals from subsequent parenteral challenge with homologous VEEV strains.^{201,212,221} Passive transfer

of nonneutralizing, anti-E1 monoclonal antibodies directed against appropriate epitopes is also protective against SINV,²⁰⁰ WEEV,¹⁹⁸ and VEEV.²⁰¹ Monoclonal antibodies specific for the E3 protein of VEEV IAB do not neutralize VEEV IAB TrD *in vitro*; however, they inhibit VEEV IAB TrD production in infected cells and protect against intraperitoneal challenge with VEEV IAB TrD after passive transfer in mice.¹⁰⁶ In contrast, for the respiratory route of infection, uniform protection was not observed after passive transfer of hyperimmune serum to hamsters¹⁹⁸ neutralizing monoclonal antibodies to mice,²²² suggesting that either additional immune mechanisms or the presence of protective antibodies along the respiratory tract may be needed. The time between the administration of immune serum and virus exposure may also be relevant. Protection of mice from intracerebral inoculation with WEEV was observed if immune serum was given no more than 3 days before virus exposure.^{223,224} Similarly, monkeys passively immunized with horse antiserum to EEE or WEE resisted intranasal challenge from homologous virus 24 hours later, but they were unable to resist a second challenge with the same virus 7 weeks later.²²⁵ However, as the immune serum given in both studies was xenogeneic, the loss of protective capacity was presumably related in part to active clearance of the immune serum by the recipients.

The effect of administering immune serum to animals after the establishment of intracerebral infections has also been evaluated. Several studies using different alphaviruses demonstrated at least partial protection if the immune serum was administered within 24 hours of infection.²²³⁻²²⁸ Other researchers have suggested that postinfection serum transfer may also cause a more severe pathology, or may merely delay the onset of disease symptoms.⁴¹ Aggressive serotherapy following infections of two laboratory workers who developed acute WEE encephalitis resulted in the survival of one patient,³⁹ but was ineffective in the second patient.²²⁵

In an EEE outbreak in New Jersey in 1959, 22 of 32 diagnosed patients died. Most patients had demonstrable antibody during the onset or progression of encephalitis, and neutralizing antibody titers in sera from patients who died were generally similar to those observed in patients who recovered.²²⁹ This finding, coupled with animal studies indicating that transfer of virus-neutralizing anti-sera was unable to prevent progression of disease if infection of the brain was firmly established as described above, suggests that serotherapy would be an ineffective means of treatment for these virus infections unless initiated early in the course of disease.

TABLE 20-4
VACCINES AVAILABLE FOR VEE, EEE, AND WEE VIRUSES

Vaccine	Form/Strain	Dose (mL)/ Route of Administration	Responding Schedule	Booster Dose/%	Duration*	Route
VEE (TC-83) Attenuated	TrD	0.5 mL/sc	Day 0	82%	92%	C-84/sc
VEE (C-84) [†]	Inactivated TC-83	0.5 mL/sc	After TC-83	76% NR [‡] 100% WT [§]	60% 100%	0.5 mL/sc
EEE	Inactivated PE-6 [‡]	0.5 mL/sc	Days 0, 28	58%	75%	0.1 mL/id
WEE	Inactivated CM-4884 [‡]	0.5 mL/sc	Days 0, 7, 28	50%	20%	0.5 mL/sc

*% of responders whose virus-neutralizing titers persist for at least 1 year

[†]current IND protocols specify use of C-84 only as a booster vaccine

[‡]TC-83 nonresponders

[§]TC-83 responders given C-84 to boost waning titers

[‡]laboratory designation

EEE: Eastern equine encephalitis

id: intradermal

IND: investigational new drug

sc: subcutaneous

TC: cell culture

TrD: Trinidad donkey

VEE: Venezuelan equine encephalitis

WEE: Western equine encephalitis

Active Immunization

Investigational New Drug Vaccines

Although no vaccines exist against the encephalitic alphaviruses that are licensed for use in humans, the US Army has developed vaccines that are currently used under IND status to protect at-risk personnel including the live-attenuated VEEV vaccine TC-83 and inactivated vaccines for VEEV (C-84), EEEV, and WEEV. The characteristics of these vaccines and the responses induced in human vaccinees are summarized in Table 20-4.

Live Vaccines

The TC-83 VEEV vaccine, which was developed in 1961 by serial passage of the virulent TrD strain in fetal guinea pig heart cells,²³⁰ is administered subcutaneously at 1×10^4 to 2×10^4 plaque-forming units per 0.5 mL dose. The vaccine was used initially in laboratory and field personnel at risk for exposure to VEEV,²³¹ and more than 6,000 people received the vaccine between 1964 and 1972.²³¹ For reasons that remain unclear, approximately 20% of the people who receive TC-83 fail to develop a detectable neutralizing antibody response and presumably would not be protected if exposed to the virus. Another 20%

to 25% of vaccine recipients experience clinical reactions ranging from mild transient symptoms to fever, chills, sore throat, and malaise in some cases sufficient to require bedrest.^{230,232} However, for recipients who respond with postvaccination titers of at least 1 per 20, long-term follow-up studies have shown that titers persist for several years.²³³ In humans, documented vaccine-breakthrough infections have been attributed largely to exposure to heterologous, enzootic strains of VEEV.^{37,61,62} Although pregnant mares were not adversely affected by TC-83,²³⁴ pregnant women are advised not to receive the TC-83 vaccine because wild-type VEEV may have been associated with spontaneous abortions or stillbirths during epidemics in Venezuela in 1962 and 1995.^{27,121}

In animals, TC-83 vaccination protects hamsters from a lethal VEEV subcutaneous or aerosol challenge,¹³⁵ although up to 20% of hamsters may die from reactions to the vaccine.^{127,235} Subcutaneous immunization of monkeys with the vaccine produces neutralizing antibody responses and protection from virulent VEEV delivered by peripheral or intranasal challenge.¹³⁴ However, TC-83 provides only partial protection against aerosol challenge in outbred mice.¹³⁹ TC-83 has been extensively administered to horses, burros, and mules, in part because large numbers of equids were vaccinated during the 1969–1970 and 1995 epizootics.^{236,237} TC-83 immunization produces febrile

responses and leukopenia in some equids,^{238,239} but neutralizing antibody responses to homologous (serotype IAB) virus eventually develop in 90% of these animals.^{238,240} Although it was difficult to accurately assess vaccine efficacy under the conditions of an ongoing epizootic, herds of animals known to have been immunized at least 2 weeks before any disease occurrence in the area did not sustain any VEEV-related deaths, whereas unimmunized herds experienced up to 60% mortality rates.²³²

The phenomenon of vaccine interference, in which prior immunity to heterologous alphaviruses inhibits vaccine virus replication and subsequent immune responses, is an unresolved problem with the use of TC-83 and presumably with other live-attenuated alphavirus vaccines. This occurrence has been observed in horses,^{241,242} in which preexisting antibodies to EEE and WEE may have interfered with TC-83 vaccination. Interference has also been observed in humans, in which preexisting immunity to a live alphavirus vaccine inhibited effective subsequent immunization with a second, different alphavirus vaccine.²⁴³ However, a recent study found no evidence for interference when vaccines for VEE, WEE, and EEE were administered simultaneously to NHPs. Interference may greatly depend on the nature of the vaccine and the virus strain(s) selected for both the vaccine and in vitro neutralization studies.²⁴⁴

Inactivated Vaccines

Early attempts to develop an inactivated VEEV vaccine resulted in preparations that contained residual live virus and caused disease in 4% of those who received it.^{226,245} Development of a formalin inactivated TC-83 VEEV vaccine (C-84) was initiated because of the problems associated with incomplete inactivation.²⁴⁶ Initial clinical trials with the C-84 inactivated vaccine were begun in 1976 in 14 volunteers previously immunized with TC-83, and subsequently in 14 naive volunteers.²⁴⁷ The vaccine was found to be safe and elicited only mild tenderness at the injection site. Although C-84 was immunogenic, three doses were required to maintain detectable neutralizing antibody titers in recipients. A subsequent study has shown that most of the TC-83 nonresponders and all of the individuals with waning titers responded to a booster dose of C-84 with a high probability of maintaining a titer for 3 years.²³¹ However, the observation that hamsters given C-84 vaccine were protected from subcutaneous challenge but not from an aerosol exposure to VEE virus¹³⁵ raised concerns that C-84 vaccination may not protect at-risk laboratory workers from aerosol exposure. Therefore, C-84 is currently administered only as a booster immunogen.

The PE-6 strain of EEEV was passed in primary chick-embryo cell cultures, and then it was formalin treated and lyophilized to produce an inactivated vaccine for EEEV.²⁴⁸ This vaccine is administered as a 0.5-mL dose subcutaneously on days 0 and 28, with 0.1-mL intradermal booster doses given as needed to maintain detectable neutralizing antibody titers. In initial clinical trials, only mild reactions to the vaccine were observed, and immunogenicity was demonstrated.²⁴⁹ The vaccine was given to 896 at-risk laboratory workers between 1976 and 1991 with no significant clinical reactions observed. A long-term follow-up study of 573 recipients indicated a 58% response rate after the primary series, and a 25% chance of failing to maintain adequate titers for 1 year. Response rates and persistence of titers increased with the administration of additional booster doses.²³¹

A formalin inactivated WEEV vaccine was similarly prepared using the B-11 or CM-4884 virus strain, and it caused only mild clinical reactions when administered to WEEV-naive individuals, according to Phillip Pittman, when he was the former chief of the special immunization program at the US Army Medical Research Institute of Infectious Diseases in 1996. Between 1976 and 1990, 359 laboratory workers were immunized with this vaccine. Long-term follow-up studies have indicated that administration of three 0.5 mL doses subcutaneously on days 0, 7, and 28 results in a 50% response rate (neutralization titer >1:40) after the primary series. Only 20% of the recipients maintain a titer for 1 year, although this level can be increased to 60% to 70% with additional booster immunizations, according to Pittman.

As with the live-attenuated alphavirus vaccines, immune interference has also been observed after vaccinations with the inactivated alphavirus vaccines. Volunteers who received the inactivated EEEV and WEEV vaccines before receiving the live-attenuated VEEV vaccine had significantly lower rates of neutralizing antibody response than those receiving the VEEV vaccine before the EEEV and WEEV vaccines.²⁵⁰

Next Generation Alphavirus Vaccines

Significant limitations are associated with the live-attenuated VEEV and formalin-inactivated VEEV, EEEV, and WEEV IND vaccines used to protect at-risk personnel. These limitations include the re-actogenicity of the live-attenuated vaccine, the poor immunogenicity of the formalin-inactivated vaccines, and the demonstrated immune interference issues associated with these vaccines. As a result, efforts are underway using many different platforms to develop next-generation vaccines that can safely and

effectively protect against VEEV, EEEV, and WEEV; next-generation subunit, live-attenuated, inactivated, DNA, virus replicon particle, and SINV-based chimeric vaccines are all at various stages of development. Subunit vaccines consisting of glycoproteins produced in *Escherichia coli* or baculovirus expression systems have provided limited success in mouse models.^{251–254} Inactivated virus vaccines provided efficacy against aerosol challenge in mice.²⁵⁵ However, further study is required to determine the efficacy of subunit and inactivated virus vaccines in NHPs. SINV-based chimeric virus vaccines are immunogenic and protect mice against VEEV, EEEV, and WEEV.^{256–260} A recent study demonstrated a chimeric SINV–EEEV vaccine candidate protected most NHPs (82%) from lethal EEE disease following aerosol infection.²⁶¹ The US Army has extensive experience in the development of next-generation live-attenuated, DNA, and virus-like replicon particle vaccines to protect against the encephalitic alphaviruses.

V3526

The next-generation live-attenuated VEEV vaccine, V3526, was created by mutation of the furin cleavage site of PE2 in wild-type VEEV IAB combined with a second-site suppressor mutation in the E1 protein.²⁶² These mutations significantly reduced the neurovirulence of V3526 as compared to the parent clone and TC-83 in mice, NHPs, and horses, and they stabilized the attenuated phenotype.^{130,150,151,263,264} V3526 has been shown to effectively elicit protective immune responses in rodents, NHPs, and horses against lethal subcutaneous or aerosol challenges with VEEV IAB TrD as well as other VEEV subtypes and related viruses (IC, IE, and Mucambo virus).^{135,130,150,151,263} Mice were protected from aerosol challenge with VEEV IAB TrD for up to 1 year following vaccination with V3526.¹⁵¹ Furthermore, immune interference was not observed when V3526 was administered to mice previously vaccinated with other alphavirus vaccines. Based on the success of V3526 in nonclinical studies, a phase 1 clinical trial was conducted to evaluate the safety, tolerability, and immunogenicity of this vaccine candidate in humans. V3526 was immunogenic in virtually all recipients, with robust immune responses elicited after administration of a single dose of the vaccine down to doses as low as 25 plaque-forming units. However, a significant number of the vaccinated subjects experienced adverse events consistent with a viral syndrome to include headache, fever, malaise, myalgia, and sore throat.²⁶⁵ Based on these findings, clinical development of V3526 was discontinued. Gamma irradiated and formalin-

inactivated V3256 vaccines have subsequently been tested in mice, but these have not progressed beyond animal studies.^{266–268}

DNA Vaccines

DNA vaccination with plasmids that express protein antigens within cells has numerous inherent advantages as a platform for the development of next-generation vaccines. Some of these benefits include that DNA vaccines:

- can be rapidly produced using well-established Good Manufacturing Practices and without the need to propagate a pathogen or inactivate an infectious organism;
- avoid problems of preexisting immunity resulting from a lack of a host immune response to the vector backbone; and
- have been demonstrated to be safe in numerous human clinical trials.²⁶⁹

Although a DNA vaccine expressing the structural proteins (C-E3-E2-6K-E1) of VEEV IAB TrD from the wild-type genes delivered by particle-mediated epidermal delivery or “gene gun” elicited strong overall antibody responses in multiple animal species, the neutralizing antibody responses were low and only partial protection against VEEV IAB TrD aerosol challenge was observed in mice and NHPs.^{270–272} A codon-optimized DNA vaccine construct expressing the structural proteins of VEEV IAB TrD minus the capsid protein delivered by intramuscular electroporation elicited improved antibody responses, including high levels of neutralizing antibodies in multiple animal species, and it provided protective immunity against VEEV IAB TrD aerosol challenge in mice and NHPs.²⁷³ Based on these results, a phase 1 clinical trial to evaluate the safety, tolerability, and immunogenicity of this vaccine candidate in humans has been initiated. A trivalent formulation of VEEV, EEEV, and WEEV DNA vaccine constructs has also been extensively evaluated in animals to reach the goal of developing a vaccine capable of simultaneously eliciting protective immunity against VEEV, EEEV, and WEEV. Important to this goal, the immunogenicity of the combined VEEV, EEEV, and WEEV DNA vaccines was not significantly reduced as compared to the individual DNA vaccines, and protection against VEEV, EEEV, and WEEV aerosol challenge has been observed in mice and NHPs. As a result, nonclinical studies required to advance this trivalent DNA vaccine formulation into phase 1 clinical testing in humans are being conducted.

Virus Replicon Particle Vaccines

Alphavirus-based replicon systems, derived by deletion of the genes encoding the viral structural proteins from full-length genomic complementary DNA clones and replacing these with heterologous genes of interest, represent a promising method for the development of next-generation vaccines.²⁷⁴ Virus-like replicon particles (VRPs) are produced in vitro following cotransfection of cells with the replicon RNA, which express the nonstructural proteins in cis and helper RNAs, which supply the structural proteins in trans.²⁷⁵ The immunogenicity and protective efficacy of VRPs expressing VEEV, EEEV, or WEEV envelope glycoprotein genes containing the furin cleavage site mutation in PE2 have been extensively evaluated in mice and

NHPs.²⁴⁴ The VEEV, EEEV, and WEEV VRP vaccines elicited strong neutralizing antibody responses when administered individually and in combination to mice. In addition, mice receiving the individual or combined VRP vaccines were protected from respective VEEV, EEEV, or WEEV aerosol challenge up to 12 months after vaccination. NHPs receiving the individual VEEV or EEEV or the combined VRP vaccines developed strong neutralizing antibody responses and were protected against VEEV and EEEV aerosol challenge, respectively. However, the individual WEEV and combined VRP vaccines elicited low or no neutralizing antibodies against WEEV in NHPs, and incomplete protection against WEEV aerosol challenge was observed. The VEEV, EEEV, and WEEV VRP vaccines have not yet progressed beyond nonclinical studies.

THERAPEUTICS

No licensed therapeutics are available for the specific treatment of alphavirus infections in humans. However, several studies have reported the identification of compounds with in vitro efficacy against alphaviruses.^{276–280} In three of these studies, the identified compounds targeted proteins of the viral polymerase

complex.^{276–278} Two other studies identified compounds targeting host protein kinases.^{279,280} The efficacy of several of these compounds was demonstrated in rodent models of VEEV or WEEV infection^{276–279}; however, no studies have reported efficacy of any compounds in NHP models of alphavirus infection.

SUMMARY

The equine encephalitis viruses consist of three antigenically related viruses within the *Alphavirus* genus of the family *Togaviridae*: VEEV, WEEV, and EEEV. These viruses are vectored in nature by various species of mosquitoes and cause periodic epizootics among equines. Infection of equines with virulent strains of any these viruses produces a similar clinical course of severe encephalitis with high mortality. However, the clinical course following infection of humans differs. EEE is the most severe of the arbovirus encephalitides, with case fatality rates of 50% to 70%. WEEV is generally less virulent for adults, but the infection commonly produces severe encephalitis in children, with case fatality rates approaching 10%. In contrast, encephalitis is rare following VEEV infection, but essentially all infected individuals develop a prostrating syndrome of high fever, headache, malaise,

and prolonged convalescence.

Although natural infections are acquired by mosquito bite, these viruses are also highly infectious in low doses as aerosols. These viruses can be produced in large quantities using inexpensive and unsophisticated systems, are relatively stable, and are readily amenable to genetic manipulation. For these reasons, the equine encephalomyelitis viruses are considered credible biological warfare threats.

No specific therapy exists for infections caused by these viruses. A live-attenuated vaccine for VEEV (TC-83) and inactivated vaccines for VEEV, EEEV, and WEEV have been developed and are used under IND status. Although these vaccines are useful in protecting at-risk individuals, they have certain disadvantages, and improved vaccines are being developed.

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