Venezuelan equine encephalitis

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VENEZUELAN EQUINE ENCEPHALITIS*

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■ **Abstract** Venezuelan equine encephalitis virus (VEEV) remains a naturally emerging disease threat as well as a highly developed biological weapon. Recently, progress has been made in understanding the complex ecological and viral genetic mechanisms that coincide in time and space to generate outbreaks. Enzootic, equine avirulent, serotype ID VEEV strains appear to alter their serotype to IAB or IC, and their vertebrate and mosquito host range, to mediate repeated VEE emergence via mutations in the E2 envelope glycoprotein that represent convergent evolution. Adaptation to equines results in highly efficient amplification, which results in human disease. Although epizootic VEEV strains are opportunistic in their use of mosquito vectors, the most widespread outbreaks appear to involve specific adaptation to *Ochlerotatus taeniorhynchus*, the most common vector in many coastal areas. In contrast, enzootic VEEV strains are highly specialized and appear to utilize vectors exclusively in the Spissipes section of the *Culex* (*Melanoconion*) subgenus.

INTRODUCTION

Of the New World alphaviruses (Togaviridae: *Alphavirus*), Venezuelan equine encephalitis virus (VEEV) is the most important human and equine pathogen. VEEV has caused periodic outbreaks of febrile and neurological disease, primarily in Latin

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Figure 1 Map showing locations of VEE epizootics since the virus was isolated in 1938, along with virus subtypes implicated as the etiologic agents.

America, during the past century (Figure 1). Many outbreaks have involved tens to hundreds of thousands of equine and human cases, have spread over large geographical regions, and have lasted up to several years. Recent outbreaks in Mexico and South America demonstrated that VEE is a re-emerging disease. VEEV is also a highly developed biological weapon amenable to use in warfare or terrorism (53). The current emphasis on biological defense has therefore renewed interest

in VEEV, both as a naturally emerging pathogen and as a terrorist agent that could be introduced artificially to cause widespread disease.

History of VEE

Because equines remain important in Latin America for agriculture and transportation, VEE outbreaks have had profound social and economic effects in addition to direct effects on human health (55, 122, 127). VEE was first recognized as a disease of horses, mules, and donkeys in northern South America during the 1930s. There is no clear evidence from epidemiological records of outbreaks during the nineteenth century (122), and phylogenetic estimates derived from sequences of VEEV strains implicated in early outbreaks indicate that they probably evolved in the early twentieth century (82). After 1938, major VEE outbreaks occurred periodically until 1973, with interepizootic periods of roughly 10 years without any activity (64, 122). The source of the VEEV strains that caused outbreaks was therefore the subject of considerable research and remains an important topic in arbovirology. One VEE outbreak spread through Central America, Mexico, and Texas during 1969–1972, involving tens of thousands of equines and people. Between 1973 and 1992, no VEE was documented, prompting speculation that variety IAB and IC epizootic VEE viruses had become extinct (122). However, several recent outbreaks (73, 86, 131) underscore the continued natural threat of VEE in the Americas (Figure 1).

Early VEE Outbreaks

The first widely recognized VEE outbreak appeared in the central river valleys of Colombia during 1935, although epidemiological reports suggest that outbreaks may have begun during the 1920s (118). One year later the outbreak spread into the Guajira Peninsula of northern Colombia and Venezuela, a desert environment populated by large numbers of mosquitoes following infrequent rainfall. The Guajira is also inhabited by feral donkeys that serve as efficient amplification hosts and are virtually impossible to vaccinate, probably the principal reason that the Guajira has been the epicenter of many VEE outbreaks. From 1936 to 1938 the VEE outbreak spread across northern Venezuela and in 1943 appeared on the Island of Trinidad, where early vaccination efforts may have contributed to its containment. During these early outbreaks, human disease due to VEEV undoubtedly occurred. However, the connection between equine and human disease was not established until the 1950s and 1960s, when human disease was documented in Colombia (93) and VEEV was isolated from human cases of febrile illness and fatal encephalitis in northern Venezuela (14). Some of the largest outbreaks on record occurred during the 1960s in central Colombia, where over 200,000 human cases and more than 100,000 equine deaths were estimated (50). Other major outbreaks involved the Pacific coast of Peru during the 1940s (64, 122).

Identification of the Etiologic Agent of VEE

The etiological agent of VEE, VEEV, was first isolated in 1938 from the brains of fatal equine cases in Yaracuy State, Venezuela. Between 1938 and 1956, only epizootic VEEV strains, later classified as antigenic subtype IAB, were isolated in northern South America. Then, beginning in the late 1950s, VEEV and related VEE serocomplex virus strains were isolated in Central America (56, 97), South America (103), Mexico (95), and Florida (18) from sylvatic and swamp habitats in the absence of equine disease. Although these viruses utilize mosquito vectors in a manner similar to their epizootic VEEV relatives, they infect primarily small mammalian reservoir hosts and are not associated with equine disease. Humans were shown to become infected with these enzootic VEEV and VEE complex strains, with occasional fatal cases documented (56, 137). Later antigenic studies (134) demonstrated that these enzootic viruses, along with the epizootic variants, comprise a serocomplex of related alphaviruses. The VEE complex now comprises 14 subtypes and varieties and includes 7 different virus species (129) (Table 1).

Vaccine Development and Use

Soon after the first isolation of VEEV in 1938, equine vaccines were made from experimentally infected mouse brain and other animal tissues containing high titers of the virus. These vaccines, inactivated with formalin, were made in Venezuela, Peru, Trinidad, and probably other locations from the only serotype of VEEV isolated from 1938 to 1957, subtype IAB. However, such inactivated viral vaccines generally induce short-lived immunity, and equine vaccination in South America probably had a limited effect on preventing disease. Moreover, genetic studies of subtype IAB VEEV strains isolated during outbreaks from 1938 to 1973 (58), phylogenetic relationships among IAB viruses (130), and the isolation of live virus from some vaccinated humans after administration of "inactivated" vaccine preparations (111) suggest that many if not all outbreaks during this era were initiated by the use of incompletely inactivated vaccines.

Since the early 1970s, inactivated vaccines have not been produced from wild-type VEEV strains, and epizootic IC strains were never used for vaccine production. Therefore, vaccines are not the explanation for recent subtype IC outbreaks (86, 131). In 1961 the development of a live-attenuated vaccine strain, TC-83, was achieved by passaging the virulent subtype IAB strain, Trinidad donkey, 83 times in guinea pig heart cell cultures (8). This vaccine was used first in Colombia in 1968, and more extensively during the 1969–1971 Central America–Mexico-Texas outbreak, and subsequently was shown to be safe and effective during experimental equine trials (120). Currently, both live and inactivated [usually multivalent formulations combined with eastern (EEEV) and western equine encephalitis viruses (WEEV)] versions of TC-83 are used to vaccinate equines. The live-attenuated version is far superior in areas of Latin America at high risk for VEE outbreaks owing to the faster and longer-lasting immunity elicited (probably lifetime). Live

TABLE 1 VEE antigenic complex viruses

Subtype	Species	Variety	Transmission pattern	Equine virulence	Location	Vector
I	VEE virus	AB	Epizootic	Yes	Central, South, and North America	Mammalophilic mosquitoes
	VEE virus	C	Epizootic	Yes	South America	Mammalophilic mosquitoes
	VEE virus	D	Enzootic	No	Central and South America	*C.* (*Mel.*) *aikenii s.sl* (*ocossa*, *panocossa*) (41, 42); *vomerifer*, *pedroi*, *adamesi* (34)
	VEE virus	E	Enzootic	Variable	Central America, Mexico	*C.* (*Mel.*) *taeniopus* (25)
	Mosso das Pedras virus	F	Enzootic	Unknown	Brazil	Unknown
II	Everglades virus		Enzootic	No	Southern Florida	*C.* (*Mel.*) *cedecei* (17, 19, 132)
III	Mucambo virus	A	Enzootic	No	South America	*C.* (*Mel.*) *portesi* (3, 26)
	Tonate virus	B (also Bijou Bridge virus)	Enzootic	Unknown	South and North America	Unknown, *Oeciacus vicarius*[b] (cliff swallow bug) (69)
	Mucambo virus	C (strain 71D1252)	Enzootic	Unknown	Western Peru	Unknown
	Mucambo virus	D (strain V407660)	Enzootic	Unknown	Western Peru	Unknown
IV	Pixuna virus		Enzootic	Unknown	Brazil	Unknown
V	Cabassou virus		Enzootic	Unknown	French Guiana	Unknown
VI	Rio Negro[a] virus		Enzootic	Unknown	Northern Argentina	*C.* (*Mel.*) *delpontei*[b]

[a]Revision approved by the International Committee on the Taxonomy of Viruses.

[b]Preliminary vector incrimination based only on virus isolation.

TC-83 has also been used for vaccination of laboratory personnel at risk of occupational exposure, although human vaccination is accompanied by a high rate of adverse reactions and failures to seroconvert; a formalin-inactivated version of TC-83, C-84, is administered to nonresponders (78). Availability for civilians of these vaccines from the U.S. Army Special Immunizations Program has become highly restricted during recent years. A new, genetically engineered VEEV vaccine strain, 3526, appears to be superior to TC-83 in murine studies (67) and may eventually replace TC-83 if human trials corroborate its improved safety and immunogenicity.

Recent VEE Outbreaks

Following a 19-year hiatus with no confirmed VEE activity, several outbreaks have occurred during the past decade. The first began in December of 1992 in Trujillo State of western Venezuela (86). The epicenter was near a recently constructed reservoir and later spread discontinuously to the western shore of Lake Maracaibo in July of 1993. Twenty-four equine and four human cases were documented, although the actual number was undoubtedly much higher. Unlike most extensive outbreaks, the 1992–1993 epizootic/epidemic did not extend into the nearby Guajira Peninsula of Venezuela or Colombia. Experimental equine infections with subtype IC VEEV strains isolated from this outbreak demonstrated that horses efficiently amplify these strains, consistent with a typical epizootic transmission cycle (124).

Also in the summer of 1993, a small equine outbreak was described in Pacific coastal communities of Chiapas State in southern Mexico, involving 125 documented equine cases with 63 deaths (73). Three years later, from June to July 1996, another equine epizootic occurred in adjacent Oaxaca State, involving 32 horses with 12 deaths. Human cases were not detected during either outbreak, although recent human serosurveys and virus isolations from sentinel animals in the affected regions indicate that VEE is endemic (J. Estrada-Franco & S.C. Weaver, unpublished data). Vaccination (TC-83) of 38,000 (1993) and 16,000 (1996) horses, limitations on the transport of equines, and insecticide spraying were used to control the outbreaks, and bans on the importation of horses into the United States were instituted. Since 1996, sporadic cases of equine encephalitis have occurred in Mexico and Central America but remain unconfirmed by virus isolation or specific serology.

One of the largest VEE epizootics and epidemics on record, involving an estimated 75,000 to 100,000 people, occurred in 1995 (131). Cases were detected first in eastern Falcon State, Venezuela, in April and then in Carabobo, Yaracuy, and Lara States during July. By mid-July the epidemic had spread into northeastern Zulia State, and in August a major epidemic occurred in rural areas of the Guajira Peninsula, both in Venezuela and Colombia. Rainfall was unusually heavy during July, August, and September, and mosquito collections in affected communities yielded large numbers of *Ochlerotatus* (formerly *Aedes*) *taeniorhynchus*,

Psorophora confinnis, and *Anopheles aquasalis*. Emergency control measures, including restricting the movement of equines, aerial and ground insecticide applications, and equine vaccination (more than 95,000 animals in Colombia alone), may have prevented the epizootic from moving south into more populated regions of Colombia. Sporadic human and equine cases continued in central Venezuela (Trujillo, Portuguesa, Cojedes, and Guarico States) until December 1995. This outbreak was remarkably similar to one that occurred in the same regions of Venezuela and Colombia during 1962–1964 (14). Symptoms and signs of infected patients, estimated human mortality rates (∼0.5%), unusually heavy rainfall preceding the epidemic, and seasonal patterns of transmission were all similar to those reported in 1962. In addition, viruses isolated during 1995 were antigenically identical (subtype IC) and nearly genetically identical to those obtained from 1962 to 1964.

VEE COMPLEX VIRUSES

VEEV Structure and Replication

VEEV is a spherical virus 70 nm in diameter with icosahedral $T = 4$ symmetry (76) (Figure 2) and a messenger-sense, single-stranded RNA genome approximately 11,400 nucleotides in length (49, 99). At its 5′ end the genome encodes four nonstructural proteins (nsP1–4), which participate in genome replication and viral protein processing in the host cell cytoplasm (Figure 3). The 3′ end one third of the genome is colinear with a subgenomic message that is translated to produce three structural proteins: the capsid and the E1 and E2 envelope glycoproteins. Structural studies of other alphaviruses indicate that the E2 protein forms spikes on the surface of the virion, and the E1 protein lies adjacent to the host cell–derived lipid envelope (79) (Figure 2). VEEV can use the laminin binding protein as a receptor for entry into cells via receptor-mediated endocytosis (66), and passage in cell culture selects for artifactual binding to glycosaminoglycans such as heparan sulfate via mutations on the surface of the E2 protein (9, 125). However, these receptor interactions do not explain the restricted vector host range exhibited by VEEV strains. After fusion of virions with the membrane of endosomes at low pH via a hydrophobic amino acid sequence in the E1 protein, the genome is translated in the cytoplasm to generate the nonstructural polyprotein. Viral genome replication takes place on the cytoplasmic surface of endosomes to produce minus-strand copies that serve as templates for genomic plus-strands and an excess of subgenomic 26S messages that encode the structural proteins. One molecule of genomic RNA interacts in the cytoplasm with 240 copies of the capsid protein to form a nucleocapsid. The envelope glycoproteins are inserted into the endoplasmic reticulum membrane and, following processing through the secretory pathway, interact with nucleocapsids at the plasma membrane to initiate budding of virus particles on the surface of cells.

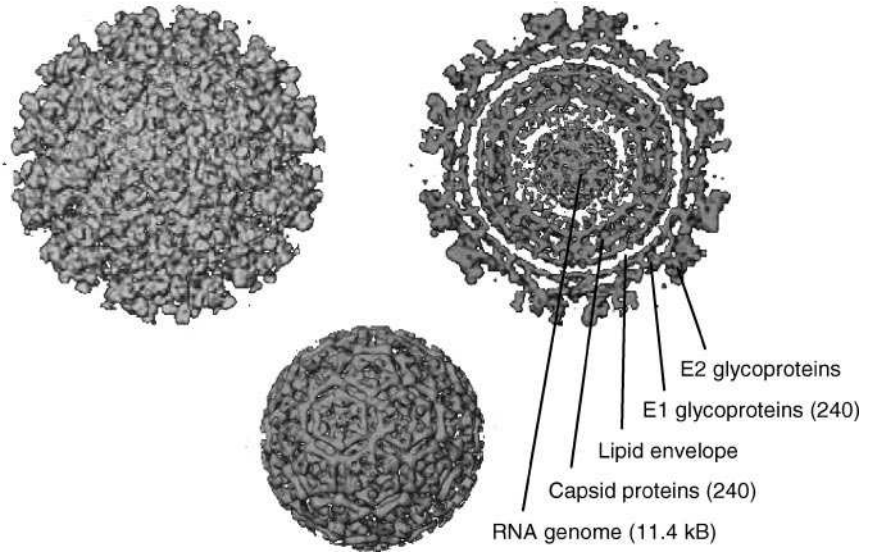

Figure 2 Cryo-electron microscopic reconstruction of the VEE virion at 14 Å resolution. (*Upper left*) External view of the virion showing the envelope glycoproteins on the surface. (*Upper right*) Cross section of the virion showing the RNA genome at the center surrounded by 240 copies of the capsid protein, the bilipid envelope derived from the host cell plasma membrane, the E1 envelope glycoprotein lying parallel to the envelope, and the E2 envelope glycoprotein forming spikes on the surface. (*Lower*) Nucleocapsid showing the capsid proteins in T = 4 icosahedral symmetry. Courtesy of A. Paredes & W. Chiu, Baylor College of Medicine, and S. Watowich & S.C. Weaver, University of Texas Medical Branch.

VEE Complex Systematics

VEE is one of 28 different virus species in the genus *Alphavirus*, family *Togaviridae* (15, 129) (Table 1) (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm). The VEE complex is one of seven different antigenic complexes of related alphaviruses and is the sister group of EEEV (81). Most alphaviruses are arthropod-borne viruses (arboviruses), although a few members of the genus infect fish and seals and some

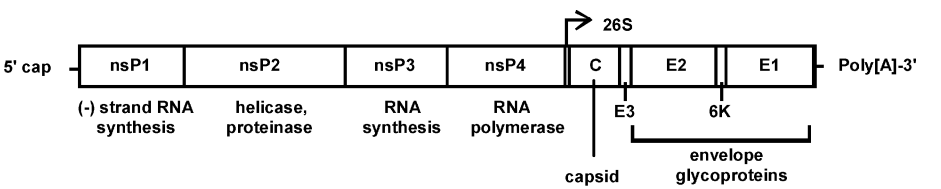

Figure 3 Organization of the VEEV genome showing encoded proteins and their major functions.

of these presumably do not utilize arthropod vectors. The other genus in the family *Togaviridae*, *Ruvibirus*, contains rubella virus, a nonvector-borne human pathogen, as its sole species.

VEEV is one of seven different species in the VEE complex. Only subtypes IAB and IC are traditionally considered epizootic strains that exploit equines as amplification hosts via the generation of high titered viremia. The remaining VEEV subtypes (ID and IE) are considered equine-avirulent, enzootic strains, although IE strains from recent Mexican epizootics appear to be equine neurovirulent but incapable of generating high-titer equine viremia (44). The remaining species in the VEE complex are also enzootic strains that generally circulate in sylvatic or swamp habitats and are considered incapable of equine amplification. In North America these include Everglades virus in southern Florida and a variant of Tonate virus, Bijou Bridge, which was isolated in Colorado from cliff swallow bugs during the 1970s but not since (69). Most if not all of the enzootic VEE complex members cause nonspecific febrile illness in people, and fatal disease has been reported for some enzootic variants (56, 137). Laboratory- (aerosol exposure) and naturally acquired human infections with enzootic VEE serotypes ID, IE, II, and IIIA have also been documented, and small epidemics involving tens to hundreds of people in the absence of equine disease have also been described (93).

Although the known distributions of the enzootic VEE complex viruses and subtypes are biased by the distribution of arbovirus surveillance programs in Latin America, they appear to be mostly nonoverlapping and extend from northern Argentina to Florida and the Rocky Mountains in North America (Figure 4). An exception is the Amazon Basin of Peru, where three distinct VEE complex virus variants circulate in the vicinity of Iquitos (subtypes ID, IIIC, and IIID) (1). Geographically delineated VEEV lineages or genotypes for subtypes ID (88) and IE (74) have also been identified, and these too appear to be nonoverlapping in their distribution. Although geographic barriers probably explain the distributions of most of these enzootic variants, an ID lineage found in Panama also circulates in Peru, with more distantly related lineages in between these countries (75).

Pathogenesis and Virulence of VEEV in Vertebrate Hosts

In equines and humans, VEEV causes a spectrum of disease ranging from inapparent to acute encephalitis. Enzootic VEE strains in subtypes I-E, II, III, and IV are avirulent for equines and generally produce only low titered viremia and little or no illness (55, 121). However, at least some of the enzootic viruses can be pathogenic for humans (39) and have caused fatal disease (56, 137). Recent epidemiological studies of febrile human illness in Iquitos, Peru, indicate that some enzootic VEEV strains may be less pathogenic than epizootic variants. In over 6600 cases of dengue-like illness studies, enzootic VEE complex viruses were the etiological agents of 166, yet no neurological disease or fatalities were detected (D. Watts, personal communication). However, only 10 of the confirmed cases occurred in children owing to intentional bias in the sampling and possibly reflecting

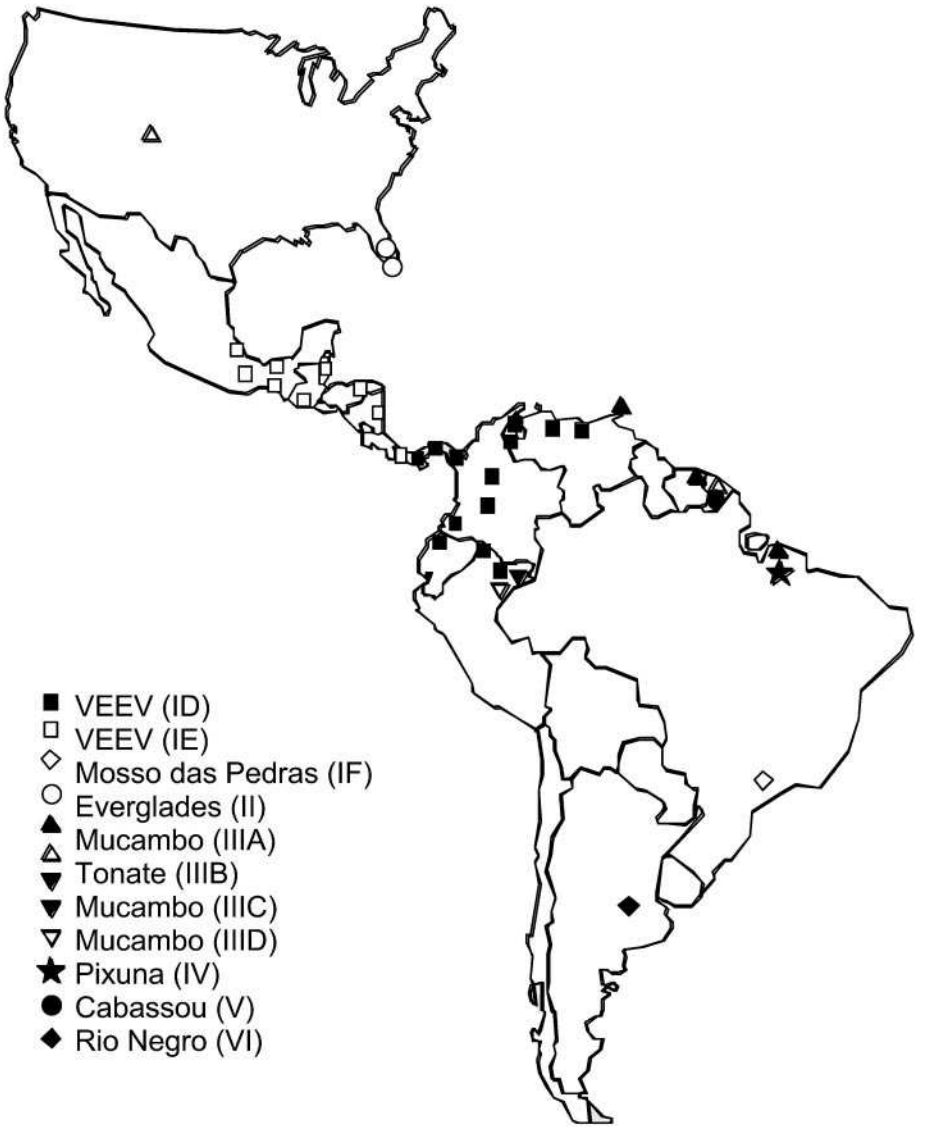

Figure 4 Map showing the known distribution of the VEE complex viruses. Subtypes are indicated in parentheses following species.

occupational exposure. In contrast, epizootic IAB and IC viruses may be more virulent for both humans and equines. Equine mortality rates during epizootics have been estimated at 19%–83%, whereas human fatalities occur less frequently, with neurological disease appearing in only about 4%–14% of cases (55, 122). VEE occurs in all human age groups, with no sex bias observed during most

outbreaks. Children are more likely to develop fatal encephalitis and suffer permanent neurological sequelae than adults. VEEV also infects the fetus in pregnant women and causes birth defects as well as spontaneous abortions and stillbirths.

In equines, generalized signs usually appear about 2–5 days after infection with epizootic VEEV, including fever, tachycardia, depression, and anorexia (55, 121, 124). Some or most animals go on to develop encephalitis 5–10 days after infection, with signs of circling, ataxia, and hyperexcitability. Death usually occurs about one week after experimental infection. Encephalitis and death are correlative with the magnitude of equine viremia, but even equine-avirulent enzootic strains produce lethal encephalitis when inoculated intracerebrally. This suggests that virulence is related to the ability of VEEV to replicate extracerebrally and spread to the brain rather than to innate neurovirulence. Resistance to murine interferon (IFN)-∂/β has been suggested as a determinant of epizootic potential (54, 107). However, studies of strains from the 1992 and 1995 Venezuelan outbreaks suggest that IFN-∂/β sensitivity is not related to the emergence of equine virulence (M. Anishchenko, S. Paessler & S.C. Weaver, unpublished data). Attenuation of the TC-83 vaccine strain was accompanied by an increase in IFN-∂/β sensitivity; this change was mapped to an E2 glycoprotein amino acid change that is augmented by a 5′-noncoding nucleotide change (107).

In human VEEV infection the incubation period is usually 2–5 days (55). Most infections are apparent, and symptoms appear abruptly, including malaise, fever, chills, and severe retro-orbital or occipital headache. Myalgia typically centers in the thighs and lumbar region of the back. Signs usually include leukopenia, tachycardia, and fever and are frequently accompanied by nausea, vomiting, and diarrhea. Signs and symptoms of central nervous system involvement occur less frequently and typically include convulsions, somnolence, confusion, and photophobia. Acute disease usually subsides 4–6 days after onset, followed by asthenia of several weeks duration. Occasionally, illness is biphasic with recurrence 4–8 days after onset. A small proportion of human cases proceed to stupor and coma and are sometimes followed by death. Lethal human VEE (generally less than 1% of cases) is accompanied by diffuse congestion and edema with hemorrhage in the brain, gastrointestinal tract, and lungs (28). Meningoencephalitis associated with intense necrotizing vasculitis and cerebritis is also observed in some patients. As with equines and laboratory rodents, a striking depletion of lymphocytes occurs in the lymph nodes, spleen, and gastrointestinal tract.

Following experimental infection with VEEV, nonhuman primates develop a nonspecific febrile illness similar to that in most human cases. Domestic rabbits, goats, dogs, and sheep also suffer fatal disease during VEE epizootics (55). Laboratory rodents including hamsters and mice are highly susceptible to infection with all VEE complex variants and suffer fatal disease with some subtypes (122). Guinea pigs are killed by epizootic IAB and IC viruses and some enzootic ID strains, but not by other enzootic subtypes (some ID and all IE, II, III, and IV strains tested) (94). In experimentally infected equines and rodents, VEEV causes severe myeloid depletion in bone marrow and lymphocyte destruction in lymph

nodes and spleen (27, 48, 94). Encephalitis is accompanied by a wide range of histopathology, from mild neutrophilic infiltration to neuronal degeneration, necrotizing vasculitis, and Purkinje cell destruction. In mice, VEEV appears to reach the brain via the olfactory nerve, seeded by viremia (27). Natural reservoir hosts become viremic but generally show no detectable disease after experimental infection (11, 135, 136).

Infection and Dissemination of VEEV in Mosquito Vectors

Biological transmission of arthropod-borne viruses generally involves initial infection of the mosquito midgut following ingestion of a viremic blood meal (126). Posterior midgut epithelial cells are believed to become infected first, followed by dissemination into the hemocoel and infection of secondary organs and tissues including the salivary glands. Virus maturation (budding) of alphaviruses in midgut epithelial cells occurs exclusively on the basal margins adjacent to the basal lamina. The mechanism of passage through the basal lamina is unknown; direct penetration seems unlikely because of the small pore size. Cytopathic effects on midgut epithelial cells by EEEV and WEEV may facilitate dissemination into the hemocoel. Although this mechanism has not been investigated with VEEV, cytopathology in salivary glands of experimentally infected *Aedes aegypti* has been detected (61). In the salivary glands, VEEV particles bud into the apical cavities and accumulate there, before salivation during blood feeding results in their passage through the salivary ducts and into the vertebrate host (126). Mosquito saliva appears to be deposited primarily in the extravascular space prior to canulation of a blood vessel (117).

TRANSMISSION CYCLES OF VEEV

Epizootic VEEV Transmission Cycles

The epizootic transmission cycle of VEEV has been studied during many outbreaks and is reasonably well understood. A feature common to all major outbreaks is the role of equines as highly efficient amplification hosts. Although the vertebrate host range of epizootic VEEV strains is wide and includes humans, sheep, dogs, bats, rodents, and some birds, major epidemics in the absence of equine cases have never occurred despite the repeated occurrence of epizootics near major cities such as Maracaibo. Experimentally infected equines generally develop viremia lasting 2–4 days, with typical peak titers of 10^5 to 10^7 suckling mouse LD_{50}/ml serum and occasional titers of 10^8 (55, 124). Humans develop similar levels of viremia but probably are incapable of efficient amplification because of their lesser exposure to mosquito bites. However, the potential for urban transmission by a species such as *A. aegypti*, which is susceptible to infection after biting humans and exhibits behavioral traits such as multiple host feeding and peridomesticity that augment its vector competence (52), should be considered as human populations continue to expand and those of equines decline in Latin America.

Epizootic VEEV Vectors

Epizootic, subtype IAB and IC strains of VEEV are opportunistic in their use of mosquito vectors during outbreaks. Field studies have indicated that more than one principal vector species can be involved in transmission during a given outbreak (92, 110). Although susceptibility to infection is a prerequisite for biological transmission and some species are almost completely refractory, ecological and behavioral traits such as longevity, host preference, survival, and population size are probably more important than susceptibility differences in vectorial capacity. Some species that appear to be only moderately susceptible to infection have been incriminated as important vectors during outbreaks.

On the basis of traditional criteria such as (*a*) demonstration of feeding or other effective contact with pathogen's host, (*b*) association in time and space of the vector and pathogen, (*c*) repeated demonstration of natural infection of the vector, and (*d*) experimental transmission of the pathogen by the vector, several mosquito species have been incriminated as VEEV vectors during epizootics. *P. confinnis* and *P. columbiae* (previous not distinguished) were probably important vectors during outbreaks in northern South America and in the 1971 epizootic/epidemic in northern Mexico and Texas (110). *Ochlerotatus sollicitans* also exhibited extremely high infection rates in coastal areas of Mexico and Texas in 1971 (110) and is capable of laboratory transmission following high-titer blood meals (116). *O. taeniorhynchus* may be the most important epizootic vector in South America. This species is abundant in coastal areas including the Guajira Peninsula, where many of the largest outbreaks have occurred, and virus isolations and susceptibility studies have documented its role in transmission (59, 110, 116). *Culex* (*Deinocerites*) spp. may also be VEEV vectors in coastal areas (47).

Nonmosquito arthropods have also been implicated as VEEV vectors. Black flies were probably important mechanical vectors (arthropods that transmit via contaminated mouth parts without virus replication) during the 1967 central Colombian epizootic (92). Ticks, including *Amblyomma cajennense* (Acari: Ixodidae) (62) and *Hyalomma truncatum* (63), are susceptible to infection by enzootic and epizootic VEEV strains. However, trans-stadial transmission, required for biological transmission, is inefficient. Mites are capable of mechanical transmission of VEEV (30).

Enzootic VEEV Transmission Cycles

Sylvatic rodents in the genera *Sigmodon*, *Oryzomys*, *Zygodontomys*, *Heteromys*, *Peromyscus*, and *Proechimys* are believed to be the principal reservoir hosts of most enzootic VEE complex viruses because they are frequently infected in nature, have high rates of immunity, and develop moderate to high titered viremia (55, 122). Spiny rats (*Proechimys semispinosus*) and cotton rats (*Sigmodon hispidus*) are the principal reservoir hosts of enzootic subtype ID viruses in Panama (46), and another species of spiny rat (*Proechimys chrysaeolus*) is the principal reservoir host in central Colombia (5). *S. hispidus* and *Peromyscus gossypinus* are probably the

most important reservoir hosts of Everglades virus in Florida (19, 65). Comparative studies in Venezuela and Colombia demonstrated a strong correlation between spiny rat (*Proechimys chrysaeolus* in Colombia) populations and levels of VEEV circulation (5). Experimental infections of *P. semispinosus* and *S. hispidus* with a Panamanian ID strain yielded 4–5 days of viremia, with peak titers of about 10^5 and 10^7 PFU/ml, respectively (135). The virus was also detected in the throat, but rodent-to-rodent transmission was not observed. *S. hispidus* appeared ill for 2–4 days, although no fever was detected; *P. semispinosus* showed no adverse signs of infection. Other mammals such as opossums (*Didelphis marsupialis*) are also frequently infected, and bats and shore birds may be involved in dispersal of enzootic viruses.

Enzootic VEEV Vectors

THE SUBGENUS *MELANOCONION* AS VEEV VECTORS A major impediment to ecological and epidemiological studies of enzootic VEEV is the difficulty of identifying and classifying the mosquito vectors. The majority of arboviruses circulating in Neotropical forests are transmitted by members of the genus *Culex*, subgenus *Melanoconion* (41, 127), a diverse and taxonomically difficult group of 156 recognized species that occurs throughout the Neotropics (a few occur in subtropical and temperate areas) (38, 51, 77, 89, 90). Seven species are proven vectors of VEE complex viruses (Tables 1 and 2), and others are likely vectors of EEEV, as well group C and Guama group arboviruses in the family *Bunyaviridae*. Most studies of enzootic VEEV ecology have incriminated a single species as the principal vector in a given location (55, 122), although three species were implicated in a single Colombian forest (34).

The subgenus *Melanoconion* is divided into the Melanoconion and Spissipes sections (89, 105); the latter taxon includes most vectors of Neotropical arboviruses, including all known vectors of enzootic VEE, EEE, and WEE complex viruses in Latin America (84, 100) and most vectors of group C and Guama group bunyaviruses (104). The restriction of most *Melanoconion* arbovirus vectors to the Spissipes Section raises the question of what genetic, physiological, and/or ecological characteristics are shared by the members of this section that predispose them to transmit arboviruses.

The ecology of most *Melanoconion* species is poorly understood, partly owing to taxonomic difficulties associated with the adult females. The immature stages of many species have never been described despite concerted efforts. Adults occur primarily in tropical forests or in swamps in association with aquatic plants such as *Pistia* (41). Many adult females, especially VEE vectors in the Spissipes section, feed primarily on small sylvatic mammals (2, 113); some exhibit more opportunistic feeding behavior and readily bite humans and domestic animals (24, 36, 38). Blood-feeding periodicity is generally crepuscular or nocturnal. The preference for small mammals, the reservoir hosts of many arboviruses, may be one ecological factor that predisposes these mosquitoes to being efficient VEEV vectors.

TABLE 2 Checklist of Spissipes section [Culex (*Melanoconion*)] mosquito species

Species	Evidence of natural VEEV transmission
adamesi[a] Sirivanakarn & Galindo 1980	Subtype ID-Colombia (34)
akritos Forattini & Sallum 1995	None
cedecei[a] Stone & Hair 1968	Subtype II (Everglades virus)-Florida (19, 132)
crybda Dyar 1924	None
delpontei Duret 1969	Subtype VI (Rio Negro virus) (16)
epanastasis Dyar 1922	None
faurani Duret 1968	None
jubifer Komp & Brown 1935	None
gnomatos[b] Sallum, Hutchings & Ferreira 1997	Subtype ID-Peru (115)
ikelos Forattini & Sallum 1995	None
lopesi Sirivanakarn & Jacob 1979	None
ocossa[a] Dyar & Knab 1919	Subtype ID-Panama (42)
panocossa[a] Dyar 1923	Subtype ID-Panama (42)
paracrybda Komp 1936	None
pedroi[a] Sirivanakarn & Belkin 1980	Subtype ID-Colombia (34)
pereyrai Duret 1967	None
portesi[a] Senevet & Abbonenc 1941	Subtype IIIA (Mucambo virus) (2, 3)
ribeirensis Forattini & Sallum 1985	None
saquettae Sirivanakarn & Jacob 1981	None
simulator Dyar & Knab 1906	None
spissipes[b] Theobald 1903	Subtype ID-Venezuela (reported in error as *ferreri*) (119)
taeniopus[a] Dyar & Knab 1903	Subtype IE-Guatemala (25)
vomerifer[a] Komp 1932	Subtype ID-Colombia (34)

[a]Proven vector meeting established criteria (4).

[b]Suggested but unproven vector role based only on virus isolation or susceptibility to infection.

TAXONOMIC HISTORY OF THE SUBGENUS *MELANOCONION* Despite its public health importance, little progress has been made in the systematics of the subgenus *Melanoconion*, which underwent several changes in interpretation and taxonomic treatment from Theobald (114) (genus *Melanoconion*) until Rozeboom & Komp (87), when its subgeneric status became accepted. Due to repeated resurrections and synonymizations, the classification remains confusing. The taxonomic history has been reviewed by Sirivanakarn (105) and Pecor et al. (77).

A complete classification of *Melanoconion* awaited the comprehensive revision of Sirivanakarn (105), based on morphological characteristics, which distinguished 153 species within 3 sections: Ocellatus (4 spp.), Melanoconion (125 spp.), and Spissipes (20 spp.). Recently, Pecor et al. (77) reduced the *Melanoconion* sections to two, after excluding the four Ocellatus species without subgeneric assignment.

THE SPISSIPES SECTION Among *Melanoconion* (*sensu lato*) mosquitoes, the Spissipes group, first proposed by Galindo (40), has received the most attention owing to the inclusion of several arbovirus vectors (23, 34, 40, 105). Recently, Sallum & Forattini (89) revised the Spissipes section using adult morphological characters (with male and female keys) including the 22 species described to date.

Within the Spissipes section, several important taxonomic changes have been made recently, including *C. taeniopus* and *C. cedecei* (23) [synonymized with *opisthopus* and *annulipes* (6)], *C. aikenii s.l.* [divided into *ocossa* and *panocossa* (7)], *C. taeniopus* [with the new species emergence of *C. pedroi* (105)], *C. adamesi* (106), the resurrection of *C. pedroi* [with the new species emergence of *akritos* and *ikelos* (89)], *C. epanastasis* [with the new species emergence of *C. ribeirensis* (37)], and *C. vomerifer* [with the new species emergence of *C. gnomatos* (89)]. Recently, with the use of ribosomal DNA sequences and phylogenetic methods corroborated by morphological analyses, two cryptic species under *C. pedroi* were detected in VEEV enzootic areas of South America (72).

The evolutionary relationships of *Melanoconion* to others in the tribe Culicini have not been examined other than at higher taxonomic levels (68, 71). Recently, Navarro & Weaver (72) inferred the evolutionary relationships among the Vomerifer and Pedroi groups in the Spissipes section, two proven VEEV vector groups (34), including the twenty-three Spissipes group species described by Forattini & Sallum (38) (Table 2).

ECOLOGY OF MOSQUITOES IN THE *MELANOCONION* SUBGENUS IN COLOMBIA In Colombia, 54 species in the *Culex* (*Melanoconion*) subgenus have been identified in diverse habitats ranging from the desert-like Guajira Peninsula to the humid, tropical forests of Chocó Department (70). The abundance and diversity of many of these species are typified by a humid tropical forest in the Middle Magdalena Valley of Colombia (5), where from 1998 to 1999 an enzootic focus of subtype ID VEEV was studied intensively. Fourteen *Culex* (*Melanoconion*) species, 6 from the Spissipes section (*C. pedroi*, *C. vomerifer*, *C. adamesi*, *C. spissipes*, *C. crybda*, and *C. ocossa*) and 8 from the Melanoconion section were collected in CDC light traps. The majority of the males (2573/3009) of the males collected during the two-year study were captured during May 1999; the factors that regulate the massive emergence of these mosquitoes in the month of May are unknown and more detailed studies are needed. However, climatic variables are probably involved.

Studies of the behavior of the VEEV vector species (*C. pedroi*, *C. vomerifer*, *C. adamesi*) in the Monte San Miguel forest (5) determined that these mosquitoes have a nocturnal activity peak and remain in forest habitats (Table 3). An exception

TABLE 3 *Culex* (*Melanoconion*) spp. vector densities in and outside an enzootic focus in the Middle Magdalena Valley of Colombia

	Nocturnal captures[a]				Diurnal captures[a]			
	Forest		Pasture		Forest		Pasture	
	No. Mosquitoes	Density[a]	No. Mosquitoes	Density[a]	No. Mosquitoes	Density[a]	No. Mosquitoes	Density[a]
C.(*Mel.*) *pedroi*	21,727	603.5	69	3.6	26	0.7	1	0.05
C.(*Mel.*) *spissipes*	21,115	586.6	230	12.1	53	1.4	2	0.1
C.(*Mel.*) *vomerifer*	6492	180.3	8	0.4	9	0.2	0	0
C.(*Mel.*) *crybda*	7261	201.7	291	15.3	39	1.0	0	0
C.(*Mel.*) *adamesi*	1502	41.7	21	1.1	5	0.1	0	0
C.(*Mel.*) *dunni*	3351	93.1	59	3.1	6	0.1	0	0
C.(*Mel.*) *ocossa*	141	3.9	364	19.1	0	0	1	0.05
C.(*Mel.*) spp.	2248	62.5	8	0.42	50	1.3	0	0

[a]Number of species (females) captured in the Monte San Miguel forest, nocturnal: 36. Number of species (females) captured in pastures outside the Monte San Miguel forest, nocturnal: 19. Number of species (females) captured in the Monte San Miguel forest, nocturnal: 38. Number of species (females) captured in pastures outside the Monte San Miguel forest, diurnal: 19.

was *C. ocossa*, a vector incriminated in Panama, which had higher population levels outside the forest habitat (29.6 females/trap/night outside the forest versus 20.0 females/trap/night inside). In addition to *C. ocossa*, small numbers of all Spissipes section species (*C. pedroi*, *C. vomerifer*, *C. adamesi*, *C. spissipes*, and *C. crybda*), but few from the Melanoconion section, were collected outside the forest. Populations of the species common to the forest interior were most abundant in May and August (precipitation 245–266 mm per month), and those of *C. ocossa* were more abundant in November (precipitation 385 mm).

A study of the effect of climatic variables, including precipitation, maximum and minimum temperature, and relative humidity, on VEEV vector (*C. pedroi* and *C. vomerifer*) populations established that precipitation and relative humidity had a significant effect on both species. The lowest population densities occurred during the months with lowest mean precipitation (<165 mm), with 38–108 females/trap/night for *C. vomerifer* and 229–685 females/trap/night for *C. pedroi*. In months with more than 200 mm of precipitation the population densities rose, reaching values of 148–639 females/trap/night for *C. vomerifer* and 549–1337 for *C. pedroi* (C. Ferro, unpublished data).

Origins of Epizootic VEEV Strains

Because of the sporadic and discontinuous nature of epizootic VEE, the source of the subtype IAB and IC strains has been a major topic of research. Johnson & Martin (55) proposed five hypotheses to explain the origins of epizootic strains: (*a*) maintenance of epizootic strains in continuous, cryptic transmission cycles, (*b*) maintenance of epizootic strains in latent equine or other animal infections, (*c*) re-emergence of epizootic viruses following administration of incompletely inactivated vaccines, (*d*) maintenance of epizootic strains as minority subpopulations within enzootic virus populations, and (*e*) periodic emergence of epizootic viruses via mutations of enzootic strains. The evidence with regard to these hypotheses is as follows:

- No evidence for maintenance of IAB and/or IC viruses in continuous transmission cycles has been obtained during field studies where epizootics frequently occur. However, the isolation of subtype IC VEEV strains in Venezuela during 2000, nearly five years after the apparent end of the 1995 Venezuelan epizootic, indicates that this virus may circulate in Venezuela in a genetically static mode without apparent disease (G. Medina & S.C. Weaver, unpublished data). If confirmed, the interepizootic subtype IC transmission cycle must be studied to assess the implications for re-emergence of these strains.
- No evidence of latent or persistent VEEV infection of any vertebrate host has been reported, although WEEV, another alphavirus, can persist for several months in avian hosts (83).
- Genetic conservation and phylogenetic relationships of IAB viruses isolated

over a 35-year time span are consistent with a vaccine etiology in several outbreaks (58, 130).

- Stanick et al. (108) examined the possibility that epizootic IAB or IC viruses are present as a minority population within enzootic ID and IE isolates. Using hydroxylapatite chromatography and selection of small plaques on Vero cells, which can isolate as few as one epizootic virion in a population of 10^6 enzootic virions, they obtained no evidence of epizootic virions in 23 subtype ID strains examined.
- Periodic emergence of epizootic VEE viruses by mutation of enzootic strains (55, 122) was first supported by antigenic (134) and later genetic (57, 58, 85) similarities between epizootic and enzootic (subtype ID) VEEV. Phylogenetic analyses of all members of the VEE complex first showed a close evolutionary relationship among IAB, IC, and ID VEE viruses and indicated that epizootic viruses probably arose several times from subtype ID–like ancestors (128). More detailed phylogenetic studies have delineated six major lineages of enzootic VEEV, including five ID-like lineages and the subtype IE lineage. All the epizootic strains from major outbreaks fall into one of three clades nested within one of these lineages, which is otherwise composed of enzootic, subtype ID strains isolated in western Venezuela, Colombia, and northern Peru (Figure 5). Equine-virulent strains isolated during recent Mexican epizootics group with enzootic strains from the Pacific coast of Guatemala. These data support the hypothesis that epizootic VEEV strains have arisen on at least four occasions by mutation of enzootic strains and changes in host range (see below). Both the epizootic phenotype and the IC serotype represent examples of convergent evolution.

Control and Prevention of VEE Outbreaks

Prevention of VEE outbreaks could probably be attained by sustained equine vaccination in Colombia, Venezuela, and Mexico, where progenitors of epizootic strains are believed to circulate and where recent outbreaks have been documented. Although governments in affected countries generally provide free equine vaccination during and soon after outbreaks, these programs are rarely sustained and susceptible populations are replenished within 5–10 years. During outbreaks, equine vaccination can be effective if VEEV circulation is anticipated or recognized quickly, but governmental agencies are often slow in acknowledging epizootics because of their perceived veterinary/public heath failures. Although live-attenuated TC-83 vaccine is manufactured in Mexico, Venezuela, and Colombia and marketed throughout most of Latin America, some equines in South America are vaccinated with inactivated, multivalent alphavirus vaccines marketed in the United States. These vaccines are inferior for protection of animals and for the prevention of epizootics and epidemics because (*a*) inactivated preparations generate protective neutralizing immunity only after multiple inoculations, (*b*) immunity resulting from inactivated vaccines is short-lived and frequent boosters are required to

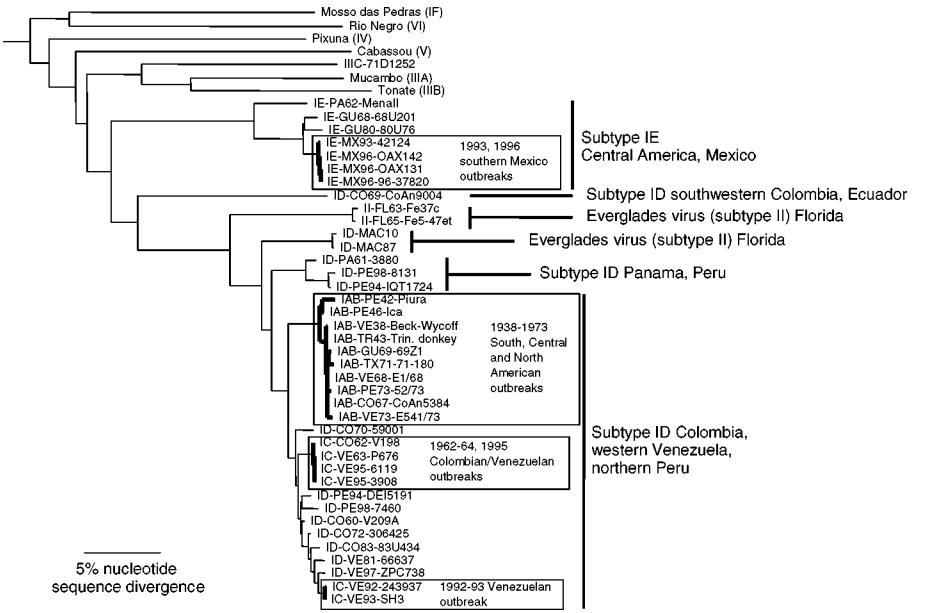

Figure 5 Phylogenetic tree derived from nucleotide sequences encoding the PE2 envelope glycoprotein using the neighbor joining method and the HK distance formula, as implemented in the PAUP 4.0 software package (112). Strains are indicated by subtype, followed by country (FL = Florida, USA) and strain designation. Major lineages of VEEV are indicated on the right, and strains isolated from epizootics are boxed and branches are in bold. Bootstrap values for all major lineages and epizootic clades were ≥98%.

maintain protection, and (*c*) once vaccinated with an inactivated preparation, equines probably do not respond appropriately to the live vaccine, requiring boosters for life to maintain immunity. Therefore, public and veterinary health officials should strongly discourage the use of these inactivated vaccines in regions of Latin America with a history of VEE. An adequate supply of live TC-83 vaccine should also be available in the United States for use in the event of a natural or terrorist-related introduction of VEEV.

Limitations on the movement of equines in affected regions have generally proved ineffective, probably because infected animals are asymptomatic for 1–3 days; owners therefore unknowingly move asymptomatic, infected animals to unaffected areas for protection. Mosquito control, principally by aerial applications of adulticides, probably has some impact on reducing transmission and may have limited the spread of VEEV during the 1971 Texas outbreak. However, reductions in rainfall also occurred coincident with a decline in VEE incidence. As with most arboviral diseases, protection of human populations relies principally on personal protection against mosquito bites by limiting physical exposure and applying repellants containing the active ingredient diethylmethylbenzamide (DEET, ≤35%

formulations recommended for adults, ≤10% for children). Permethrin can be applied to clothing to enhance protection. These protective measures are especially important for individuals who reside or work near equine herds during epizootics, or those who contact tropical forest or swamp habitats where enzootic VEEV circulates.

THE ROLE OF ADAPTATION TO HOSTS AND VECTORS IN VEE EMERGENCE

Phylogenetic studies support the hypothesis that epizootic VEEV strains arise via mutation of enzootic strains, principally ID strains from western Venezuela, Colombia and/or northern Peru, and from IE strains from the Pacific coast of Central America and Mexico (although the Mexican epizootic strains are not typical in that they do not amplify efficiently in equines) (Figure 5). These phylogenetic studies have been extended to complete genomic sequences, and the results can generate hypotheses for the mutations that mediate the host range changes in vertebrates and mosquitoes that accompany VEE emergence. When maximum parsimony methods are used to predict mutations that accompanied these four emergence events (evolution of epizootic strains), common or similar mutations can be sought to explain the convergence of the epizootic phenotype. This approach has resulted in a focus on E2 envelope glycoprotein mutations that increase the charge on the surface of the VEE virion (12, 123). Although these mutations are similar in nature to those implicated in artifactual adaptation to glycosaminoglycan binding in cell culture, analyses of low passage history epizootic strains have demonstrated that the mutations that accompany VEE emergence are positionally and functionally independent (125).

Adaptation to Equines by Epizootic VEEV

Experimental infections of equines with enzootic versus epizootic VEEV strains demonstrated many years ago that equine viremia is a major determinant of the ability to cause widespread disease. This implies that, when epizootic strains arise via mutation of enzootic progenitors, equines select for mutants with increased viremia potential and indirectly for increased virulence (Figure 6). The strong correlation between the antigenic subtype (IAB and IC) and equine viremia implies that the E2 envelope glycoprotein, the site of the major antigenic determinants, is an important determinant of equine virulence and viremia.

Thanks to advances in viral genetics over the past two decades, experimental studies using reverse genetic approaches can now be used to identify the individual genes and mutations that can adapt enzootic VEEV strains to equine amplification. Ideally, these studies would utilize in vitro markers of the epizootic phenotype and/or a small laboratory animal model that responds differentially to enzootic versus epizootic VEEV strains (like equines) following experimental infection (55, 122, 124). Plaque size is a reliable marker of the epizootic phenotype

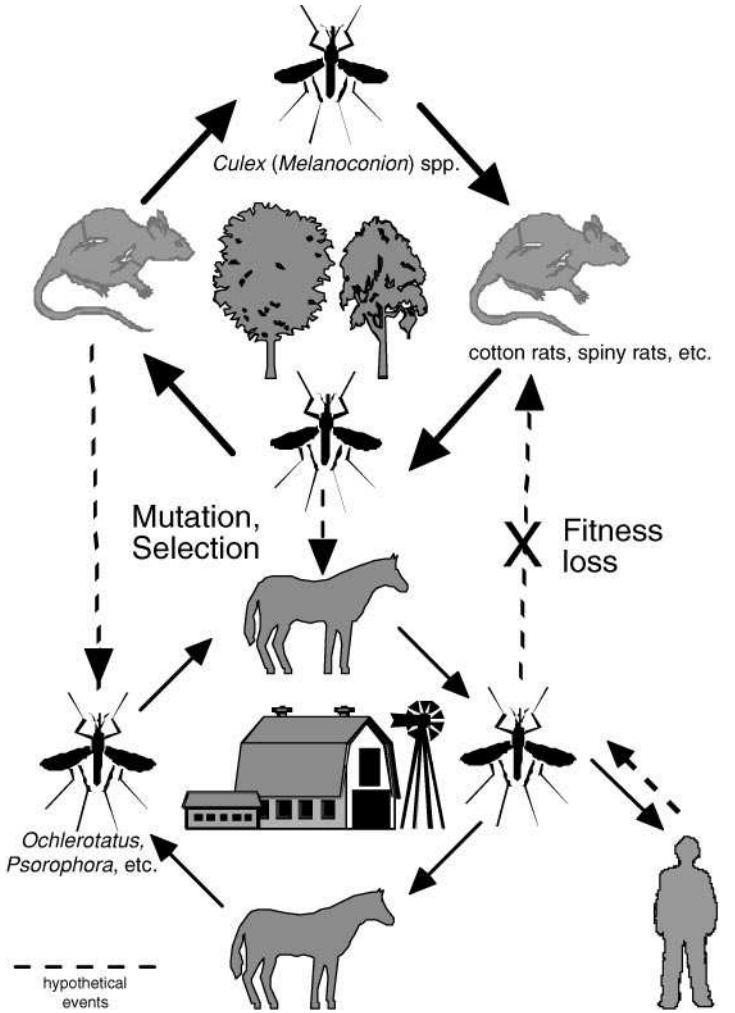

Figure 6 Cartoon depicting enzootic (*above*) and epizootic/epidemic (*below*) transmission cycles of VEEV. Arrows show hypothetical adaptation to new hosts that mediate VEE emergence from enzootic progenitors.

(epizootic strains produce small plaques on Vero cells after incubation under an unpurified agar overlay), and the positive-charge E2 mutations apparently mediate this phenotype owing to their interactions with polyanions in unpurified agar, which limit virus spread (12). Resistance to interferon (107) may be useful but is not a consistent marker (see above). Laboratory mice succumb to fatal encephalitis following infection with nearly all VEEV strains, as do hamsters. Guinea pigs suffer fatal infection with epizootic strains and survive infection with some but

not all enzootic isolates (94). Subtype IE strains are nonlethal for guinea pigs and have been used to map virulence determinants of epizootic strains.

Chimeric viruses including the nonstructural proteins of an epizootic IAB strain and structural proteins of an enzootic IE strain (IAB/IE), or reciprocal constructs (IE/IAB), show intermediate levels of virus replication and virulence for guinea pigs compared with the parental strains (80). The IE/IAB chimera produced slightly higher viremia and an average survival time two days shorter than the VE/IAB-IE virus, which suggests that the structural proteins are more important determinants of virulence. Similar results have been obtained with subtype IC and ID strains and chimeric recombinants with swapped E2 genes (I. Greene, S. Paessler & S.C. Weaver, unpublished data).

Adaptation to Epizootic Mosquito Vectors

Because the vectors implicated during epizootics generally belong to different genera from the enzootic vectors (Figure 6), adaptation for efficient transmission by epizootic vectors is also a potential mechanism that contributes to VEE emergence. If VEEV undergoes adaptation during the evolution of epizootic strains from enzootic progenitors, epizootic vectors implicated during outbreaks should show greater susceptibility to epizootic strains than to enzootic viruses closely related to their progenitors. The first evidence of this difference came from experimental studies with *O. taeniorhynchus*, a proven epizootic vector of epizootic subtype IAB and IC viruses. This species is more susceptible to subtype IAB strains than to enzootic, subtype IE strains from Guatemala (59). However, the IE strains are not closely related to the progenitors of the IAB serotype, which are believed to be ID strains from Colombia and Venezuela (Figure 5). Later studies demonstrating a greater susceptibility of *O. taeniorhynchus* to infection with IAB and IC epizootic than with ID enzootic strains more directly supported the hypothesis that adaptation to this species is an important mechanism of VEE emergence. Experiments using chimeric viruses engineered from subtype IAB, IC, and ID strains demonstrated that the E2 envelope glycoprotein is an important determinant of these susceptibility differences, suggesting that interactions with receptors on midgut epithelial cells may be involved (13).

More recent studies of the susceptibility of *O. taeniorhynchus* to infection with VEEV strains isolated during a smaller outbreak suggest that adaptation to this species may affect the size of outbreaks by facilitating efficient transmission in coastal regions. Subtype IC strains isolated during the small 1992–1993 Venezuelan outbreak, which did not extend to coastal regions inhabited by *O. taeniorhynchus* and produced a relatively small number of equine and human cases (86), were tested for their ability to infect this species. Despite the ability of the etiologic subtype IC strains to replicate efficiently in equines (124), they do not infect *O. taeniorhynchus* more efficiently than closely related enzootic ID strains believed to represent progenitors of the outbreak (D. Ortiz & S.C. Weaver, unpublished data). These data suggest that the inability of the 1992–1993 strains to adapt to *O. taeniorhynchus* may have limited the extent of the epidemic. Further studies

examining the susceptibility of three other mosquito species, including the proven epizootic vector *P. confinnis*, also show no apparent difference in susceptibility to a variety of enzootic versus epizootic VEEV strains. These studies imply that adaptation to *P. confinnis* does not accompany VEE emergence (D. Ortiz & S.C. Weaver, unpublished data).

A hypothesis to explain the apparent failure of epizootic VEEV strains to be maintained in sylvatic transmission cycles similar to those of the enzootic variants is that adaptation for infection of epizootic vectors leads to a fitness decrease in the enzootic mosquito vectors (Figure 6). Scherer et al. (98) demonstrated that enzootic vectors are in some cases more susceptible to infection by sympatric enzootic than by epizootic or allopatric enzootic variants. *C. taeniopus*, the enzootic subtype IE vector in Guatemala, is highly susceptible to infection with sympatric IE strains but relatively refractory to subtype IAB, IC, ID, II (Everglades), III (Mucambo), and IV (Pixuna) viruses. The refractoriness of *C. taeniopus* to epizootic strains may explain the inability of subtype IAB VEEV to persist in Guatemala following the 1969 outbreak. Additional experimental infections of subtype ID enzootic vectors (Table 1) with epizootic and enzootic strains are needed to test the hypothesis that adaptation to epizootic vectors reduces the fitness of epizootic strains for the ancestral enzootic vectors (Figure 6). The same fitness loss may apply to the reservoir hosts of enzootic strains when epizootic strains adapt for equine replication, and comparisons of enzootic versus epizootic VEEV infection rodent hosts are needed to evaluate this version of the hypothesis.

FUTURE DIRECTIONS OF VEE RESEARCH

Treatment and Prevention of VEE

Despite over 60 years of research on VEE, there is no licensed human vaccine or effective antiviral treatment for human or equine disease. The recent concern that VEEV may be used for biological terrorism has underscored the need to develop improved, licensed vaccines and effective antivirals. Recent advances in genetic engineering and vaccine design have been exploited to produce an improved candidate vaccine (67), and comparable efforts are needed to develop antivirals. The ability of vaccines to protect against challenge with heterologous VEE subtypes needs to be evaluated because several can cause human disease.

Mechanisms of Natural VEE Emergence

Phylogenetic and reverse genetic studies have provided strong evidence to support the hypothesis that epizootic VEEV strains can arise via mutation of enzootic strains in subtype ID. However, all three subtype IAB/IC epizootic clades appear to have arisen from only one of six major VEEV lineages. The reason(s) why the other lineages have not generated major epizootics is unknown and is an important topic of future research. One hypothesis is that the ability to generate epizootic

strains is highly dependent on the genetic makeup of the enzootic strains; the enzootic VEEV lineages that circulate in Florida, Mexico, Central America, north-central Venezuela, southwestern Colombia, Ecuador, and parts of Peru (Figure 5) may be incapable of equine adaptation via a small number of mutations. Another hypothesis is that the ecological conditions required for the onset of an epizootic do not occur in some of the areas inhabited by these enzootic strains. However, the history of VEE epizootics in Mexico, Central America, north-central Venezuela, southwestern Colombia, Ecuador, and coastal Peru argue against this hypothesis. Answering this question is important for public health in many locations including Florida, where the potential for epidemics remains unknown.

Another aspect of VEE emergence that deserves further study is the ecological mechanisms that allow newly generated epizootic strains to reach locations conducive to amplification. Most regions of South America known to harbor enzootic VEEV do not usually experience epizootics, presumably because the enzootic strains immunize equines against epizootic VEEV (124). This raises the question of how epizootic strains that may be generated via mutation in enzootic locations are translocated across this "halo" of equine immunity to locations with large populations of susceptible equines and mosquito vectors.

The possible role of flying vertebrate hosts in disseminating VEEV among enzootic foci, or in transporting epizootic viruses during outbreaks, has received little study. VEEV antibody rates are generally low in birds (55, 122), although natural avian VEEV infections were reported during a 1967 Colombian epizootic (92), and from six species of passerine and wading birds, primarily fledglings or nestlings, in Panama (43). Experimental infections indicate that some wading birds, particularly nestlings, develop viremia sufficient to infect vectors and have the potential for serving as amplifying hosts (29, 45).

The evidence for a natural role of bats in enzootic and epizootic VEEV dissemination is stronger. Virus isolations from bats have been reported in Colombia (92) and Mexico (21, 96). In Guatemala, antibodies were detected in seven species, (101), and VEEV was isolated from the blood of one *Uroderma bilobatum*. An overall seropositivity rate of 10% was estimated for the genus *Artibeus*, which suggests that these bats may serve as alternative reservoir hosts to maintain virus circulation if most terrestrial animals become immune (101). A variety of bats including vampires become viremic with moderate to high titers and shed virus into their saliva for up to 168 h following experimental infection (22, 91, 102); persistent viremia has been detected for at least 26 days at hibernating temperatures, and low viremia levels persisted for over 90 days and rose when the bats were returned to room temperature (22). These studies indicate that bats deserve further attention as potential reservoir, amplification, and/or transport hosts.

In addition to questions regarding the evolution of epizootic IAB and IC strains, their ability to persist following the apparent cessation of equine and human cases deserves attention. Evidence of VEEV circulation in 1966, two years after the apparent end of the 1962–1964 outbreak, was found in eastern Venezuela (109). Similarly, subtype IC VEEV strains were isolated in 2000 in two locations of

Venezuela, more than four years after the apparent end of the 1995 outbreak (G. Medina & S.C. Weaver, unpublished data). Because the epizootic strains have never been isolated in sylvatic cycles typical of enzootic strains, alternative transmission cycles during these interepizootic periods deserve further attention.

Molecular Systematics of VEEV Vectors

The powerful combination of DNA sequencing and phylogenetic methods has recently been used to elucidate evolutionary trends in vectors of medical importance that have long defied morphological approaches, including the genera *Anopheles* (35, 60, 133), *Aedes* (133), *Culex* (68, 72), and higher in level hierarchic resolution (10). Although morphological studies should remain a foundation, several important questions regarding arbovirus vectors can be answered using molecular systematics applied to the *Culex* (*Melanoconion*) subgenus: (*a*) Is the Spissipes section monophyletic? If so, what synapomorphic genetic, physiological, or ecological factors shared by these mosquitoes explain their important role in transmitting arboviruses? (*b*) Has VEEV coevolved (cospeciated *sensu lato*) with its *Culex* (*Melanoconion*) vectors, or has VEEV adapted to these mosquitoes independently on several occasions during the evolution of the serocomplex? (*c*) Are all arbovirus vectors in the *Culex* (*Melanoconion*) subgenus correctly identified, or are there cryptic species of medical importance? These questions are of vital importance to understanding the evolution of vector-borne diseases and their association with mosquitoes.

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