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Repeated Emergence of Epidemic/Epizootic Venezuelan Equine Encephalitis from a Single Genotype of Enzootic Subtype ID Virus

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Venezuelan equine encephalitis (VEE) epidemics and equine epizootics occurred periodically in the Americas from the 1920s until the early 1970s, when the causative viruses, subtypes IAB and IC, were postulated to have become extinct. Recent outbreaks in Columbia and Venezuela have renewed interest in the source of epidemic/epizootic viruses and their mechanism of interepizootic maintenance. We performed phylogenetic analyses of VEE virus isolates spanning the entire temporal and geographic range of strains available, using 857-nucleotide reverse transcription-PCR products including the E3 and E2 genes. Analyses indicated that epidemic/epizootic viruses are closely related to four distinct, enzootic subtype ID-like lineages. One of these lineages, which occurs in Columbia, Peru, and Venezuela, also included all of the epidemic/epizootic isolates; the remaining three ID-like lineages, which occur in Panama, Peru, Florida, coastal Ecuador, and southwestern Columbia, were apparently not associated with epizootic VEE emergence. Within the Columbia/Peru/Venezuela lineage, three distinct monophyletic groups of epidemic/epizootic viruses were delineated, indicating that VEE emergence has occurred independently at least three times (convergent evolution). Representative, complete E2 amino acid sequences were compared to identify potential determinants of equine virulence and epizootic emergence. Amino acids implicated previously in laboratory mouse attenuation generally did not vary among the natural isolates that we examined, indicating that they probably are not involved in equine virulence changes associated with VEE emergence. Most informative amino acids correlated with phylogenetic relationships rather than phenotypic characteristics, suggesting that VEE emergence has resulted from several distinct combinations of mutations that generate viruses with similar antigenic and equine virulence phenotypes.

Venezuelan equine encephalitis (VEE) has been an important human and equine disease of the Americas for much of this century. The etiologic agent, VEE virus, is a member of the VEE complex, one of three major alphavirus (*Togaviridae*: *Alphavirus*) serogroups found in the New World. At least 13 members of the VEE complex have been distinguished serologically. Subtypes II to VI, as well as subtype I, varieties D, E, and F (subtypes ID, IE, and IF), have not been associated with major epidemics and epizootics. These serotypes are called enzootic because they circulate in sylvatic or swamp habitats and only occasionally cause overt disease in humans or domestic animals (36, 41). Transmission cycles have been described for five of the enzootic subtypes (ID, IE, II, IIIA, and IIIB); all are transmitted among small mammals by mosquitoes in the subgenus *Culex* (*Melanoconion*), with the exception of Bijou Bridge virus (IIIB), which is transmitted among birds in western North America by the cliff swallow bug, *Oeciacus vicarius* (20). The remaining serotypes of VEE are classified into subtype I, varieties AB and C (36, 44). These viruses are designated epidemic or epizootic because they have been isolated only during major outbreaks involving up to hundreds of thousands of people and equines, with severe morbidity and high mortality rates, especially in horses, burros, and mules.

In equines and humans, VEE viruses cause a spectrum of disease ranging from inapparent infection to acute encephalitis. Enzootic VEE strains in subtypes ID-E, II, III, and IV are avirulent for equines, producing only low-titered viremias and little illness (35). However, at least some of these enzootic viruses can be pathogenic for humans (9) and have caused fatal disease (12). In contrast, epidemic/epizootic IAB and IC viruses are virulent for both humans and equines. Equine mortality rates have been estimated at 19 to 83%; in humans, while fatalities occur less frequently, neurological disease appears in 4 to 14% of cases (11, 36).

Epidemics and epizootics caused by VEE viruses were first recognized in Venezuela during 1936. However, retrospective examination of epidemiological data suggests that outbreaks occurred in northern South America at least since the 1920s (11, 36). Epidemics and epizootics continued sporadically through the 1960s in South America, and one affected Central America, Mexico, and Texas during 1969 to 1972. However, between 1973 and 1992, no VEE epidemic/epizootic activity was documented, prompting speculation that variety IAB and IC VEE viruses had become extinct (36). A small epidemic/epizootic in northwestern Venezuela during 1992 to 1993 (26) and a major outbreak in Venezuela and Colombia during 1995

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(42) demonstrated that these viruses remain a public and veterinary health threat.

For many years, the fundamental enigma regarding the epidemiology of VEE concerned the source of the epidemic/epizootic, variety IAB and IC viruses and their mechanism of persistence between outbreaks. The hypothesis of periodic emergence of these viruses via mutation of enzootic strains (11, 36) was supported by antigenic (44) and genetic (16, 25) similarities between epidemic/epizootic and enzootic variety ID VEE viruses. Phylogenetic analyses indicated that epidemic/epizootic viruses arose several times via mutation of variety ID-like ancestors (38). The source of a more recent (1992 to 1993) Venezuelan epidemic/epizootic was more precisely identified as a ID lineage circulating in northwestern Venezuela and northeastern Colombia (26), confirming predictions of continued epidemic/epizootic emergences based on phylogenetic data (38). A major epidemic in Venezuela and Colombia during 1995 resulted from the reemergence of a variety IC genotype implicated in a 1962–1964 outbreak in the same regions (42).

To examine in greater detail the genetic diversity of variety ID enzootic viruses and to delineate more precisely their relationship to epidemic/epizootic VEE viruses, we conducted detailed phylogenetic analyses using a large number of isolates spanning the geographic and temporal range of available strains. Our results indicate that epidemic/epizootic VEE emergence from ID-like viruses has occurred at least three times, but that only one of four ID-like enzootic lineages that we identified has been responsible for all of the outbreaks studied. VEE emergence probably relies on several different combinations of mutations which result in similar antigenic and equine virulence changes.

MATERIALS AND METHODS

Virus preparation and PCR amplification. The VEE virus strains analyzed are listed in Table 1. Most were serotyped previously by hemagglutination inhibition assay or enzyme-linked immunosorbent assay (ELISA). Virus stocks were prepared on BHK-21 cell monolayers at 37°C with a multiplicity of infection of 0.1 to 1.0 PFU per cell. After cytopathic effects were evident, RNA was extracted from 100 μl of the supernatant by using Trizol (BRL Laboratories) as described previously (2). cDNA was synthesized by using antisense primers with sequences 5′-TRCACTGGCTGAACTGTT-3′ and 5′-TGATAGGGAGTGGTGCGTAGCC-3′, designed to anneal to genome positions 9207 to 9224 and 10067 to 10088, respectively, of the Trinidad donkey strain (15), or a poly(T) oligonucleotide ($T_{19}V$) for subtype VI. For most PCR amplifications, 300 ng of antisense and sense (5′-GAGAACTGCGAGCAATGGTCA-3′, genome positions 8369 to 8388; 5′-GGAGCTTATGTCGAGATGCACCT-3′, positions 9047 to 9069) primers was added. PCR amplification of subtype VI involved the use of a poly(T) primer and a forward primer (5′-ACYCTCTACGGCTRACCTRA-3′, genome positions 7514 to 7533) to amplify the entire 26S region. PCRs were performed as described previously (2), with 30 amplification cycles as follows: heat denaturation at 95°C for 30 s, primer annealing at 49°C for 30 s, and extension at 72°C for 1 min. For VEE virus subtypes IF and III to V, the initial five cycles used an annealing temperature of 40°C; for subtype VI, all 30 cycles were done with an annealing temperature of 45°C and extension time of 10 min.

Sequencing and phylogenetic analyses. Most PCR products were sequenced directly, using an Applied Biosystems Prism automated DNA sequencing kit and sequencer according to the manufacturer's protocol. Sense primers with sequences 5′-AATTGAGGCAGTGAAGAGCGAC-3′ (genome positions 8659 to 8680) and 5′-CTGAAACTGCACCCTAAG-3′ (positions 9416 to 9433), as well as an antisense primer with the sequence 5′-CTGCCTACAGGATTAAAT-3′ (genome positions 8953 to 8967), were used in addition to those used in the PCR. PCR products from VEE virus subtype IF and III-V strains were cloned into the PCRII vector (Invitrogen), and at least two clones representing the consensus restriction digestion pattern were sequenced by using plasmid-specific primers as well as those used for PCR amplification. Primers with sequences 5′-GGTAAGATYGACAAYGACG-3′ and 5′-CGCCGTAGGTAGCTGCC-3′ (genome positions 8035 to 8053 and 8629 to 8645, respectively) were used to sequence the subtype VI virus PCR product directly. Nucleotide sequences were aligned with each other and with others determined previously (15–17, 21, 31, 42), using the PILEUP program (4). Phylogenetic analyses were conducted by using the PAUP (33) and DNAPARS parsimony algorithms, NEIGHBOR neighbor-joining and FITCH distance matrix programs implemented in the PHYLIP package (7), and the fastDNAml maximum likelihood algorithm (23). Parsimony analysis was implemented by using the heuristic algorithm and both ordered (transition/transversion = 1:4, based on previous alphavirus estimates [2, 37, 43]) and unordered characters. Sequences were added at random with 10 replications. Bootstrap resampling (6) with 200 replicates was used to place confidence values on groupings within trees.

Nucleotide sequence accession numbers. The nucleotide sequences were submitted to the GenBank library under accession no. AF004436 to AF004472, AF004852, and AF004853. Accession no. U55341, U55342, U55346, U55347, U55361, U55362, and U88632 were updated.

RESULTS

Phylogenetic analyses. PCR products of 857 nucleotides, homologous to nucleotides 8369 to 9227 of the Trinidad donkey strain (15), including the E3 gene and N terminus of E2, were analyzed by using an outgroup consisting of homologous sequences from four major genotypes of eastern equine encephalomyelitis virus (36a, 37), the sister group of the VEE complex (39). Initial analyses included prototype strains of each of the 13 VEE complex subtypes and varieties (Table 1). Phylogenetic analyses using parsimony and distance programs yielded trees differing only in the placement of subtypes IV to VI. The tree presented in Fig. 1 was constructed with the FITCH algorithm to correct branch lengths for multiple substitutions of nucleotides and shows the same branching pattern as those produced with weighted parsimony and maximum likelihood methods. The relationships reflected in this tree did not entirely agree with serological relationships represented by the current VEE complex classification (Table 1). For example, subtype III was monophyletic but subtype I was not; subtype II Everglades virus grouped with the IAB/IC/ID viruses and was more closely related to the epidemic/epizootic IAB and IC viruses than were the IE viruses. Most of these genetic relationships were consistent with those determined previously by using nsP4, E1, and 3′ untranslated sequences (38), with the exception of subtypes IF, III, V, and VI. The phylogenetic placement of these subtypes was not robust, as evidenced by low bootstrap values for internal groupings and inconsistencies in results obtained by using different phylogenetic methods. This instability of internal groupings may reflect the occurrence of several divergence events during a relatively short time period (Fig. 1).

As in the previous analyses (38), all epizootic/epidemic IAB and IC viruses consistently grouped with the enzootic, subtype ID representatives. Subtype II Everglades virus was also closely related to the IAB, IC, and ID viruses, followed by subtype IE. These phylogenetic analyses confirm the results of previous studies using polyvalent (8) and monoclonal (27, 28) antibody assays as well as structural gene sequencing (31) that demonstrated a closer relationship of subtype II viruses to subtype IAB/IC and ID than to IE viruses. Subtype IF was distantly related to the other subtype I viruses; however, when trees were manipulated to force subtype IF into the subtype I/II clade, only seven steps were added, underscoring the uncertain placement of this subtype within the phylogeny. The closer genetic relationship of Mucambo (subtype IIIA) than Pixuna (subtype IV) to the subtype IAB viruses is consistent with antigenic relationships (30, 44).

To study relationships of enzootic to epidemic/epizootic VEE viruses in greater detail, we assembled a collection of 55 isolates representing the geographic and temporal distribution of available subtype IAB, IC, ID, and II strains (Table 1). These detailed phylogenetic analyses yielded trees with only minor differences in the branching order of a few IAB and ID strains within their respective groups (Fig. 2). All trees included several distinct, robust groups of subtype IAB, IC, ID,

TABLE 1. VEE viruses used in phylogenetic analyses

Subtype	Strain	Location[a]	Date[b]	Host	Passage level[c]
IAB	Beck-Wycoff	Zulia St., Venezuela	1938	Horse	sm8, cec1
	Trinidad donkey	Trinidad	1943	Donkey	gp1, v6
	CoAn5384	Carmelo, Colombia	1967	Horse	sm1, cec2
	69Z1	Guatemala	1969	Human	sm2
	71-180	Texas	1971	Horse	sm1, h1, v1, C6/36-1
	52/73	La Libertad, Peru	1973	Donkey	sm2
IC	V-178	Cundinamarca Dept., Colombia	1961	Horse	sm1, v1
	V-198	La Guajira Dept., Colombia	1962	Human	sm1, v1
	V-202	La Guajira Dept., Colombia	1962	Human	sm1, v1
	PHO 127	Zulia St., Venezuela	1962	Human	v2
	P676	Miranda St., Venezuela	8-63	*Aedes triannulatus*	sm1, v7
	PMC Ho5	Monagas St., Venezuela	1964	Human	sm1, v1
	Panaquire	Miranda St., Venezuela	1983	Mosquito	v1
	243937	Trujillo St., Venezuela	1992	Horse	v1
	SH3	Trujillo St., Venezuela	1-9-93	Human	v1
	6119	Falcon St., Venezuela	5-24-95	Human	v1
	1327	La Guajira Dept., Colombia	10-18-95	Mosquito	Unpassaged
	1289	La Guajira Dept., Colombia	10-18-95	Horse	Unpassaged
	12563	Lara St., Venezuela	11-13-95	Human	v1
IC/ID	SH5	Trujillo St., Venezuela	1-6-93	Human	v1
ID	38851	Colombia	1957	Human	Unknown
Unknown	1385	Bolivar St., Colombia	10-25-95	Horse	Unpassaged
ID	V209A	Santander Dept., Colombia	1960	Mouse	sm2, v2
	59001	Puerto Boyaca, Colombia	7-27-70	Hamster	v1
	59145	Tibu, Colombia	1970	Hamster	sm2
	307536	Puerto Boyaca, Colombia	5-71	*Culex* (*Melanoconion*) sp.	sm2
	70U1134	Iquitos, Peru	1970	Hamster	sm1, cec1
	306425	Puerto Boyaca, Colombia	3-72	Hamster	v2
	310979	Puerto Boyaca, Colombia	7-74	Hamster	cec1
	312714	Puerto Boyaca, Colombia	11-74	*Proechymys* sp.	v2
	309752	Lozania, Colombia	1974	Human	cec1
	23647	Catatumbo, Zulia St., Venezuela	1974	Hamster	v2
	33571	Catatumbo, Zulia St., Venezuela	7-19-76	*Aedes fulvus*	sm2
	34958	Catatumbo, Zulia St., Venezuela	1976	*Culex* (*Melanoconion*) *ferreri*	sm3
	334250	Puerto Boyaca, Colombia	12-77	*Aedes fulvus*	v2
	335733	Puerto Boyaca, Colombia	8-78	Hamster	Unpassaged
	66457	Sinamaica, Zulia St., Venezuela	11-11-81	Hamster	v1, sm1
	66637	Sinamaica, Zulia St., Venezuela	11-19-81	Hamster	v1, sm1
	83U434	Rio de Oro, Colombia	6-83	Hamster	cec1, v1
	3880	Canito, Panama	4-20-61	Human	sm1, v8
	903104	Bayano, Panama	1977	*Culex* (*Melanoconion*) *aikenii* sensu lato	v2
	903843	Bayano, Panama	1984	*Culex* (*Melanoconion*) *aikenii* sensu lato	v2
	CoAn9004	Tumaco, Colombia	1969	Hamster	sm3, v1
	76V2561	Ecuador	1975	*Culex* (*Melanoconion*) sp.	sm4
	16880	Vinces, Ecuador	1976	Hamster	sm4
	16927	Vinces, Ecuador	1977	Hamster	v1
	DEI5191	Pantojas, Peru	1994	Human	v1
	IQT1724	Iquitos, Peru	1995	Human	v1
IE	MenaII	Almirante, Panama	1962	Human	sm3, v7
	68U201	La Avellana, Guatemala	1968	Hamster	sm1
	BT-2607	Almirante, Panama	1961	*Culex* (*Melanoconion*) *taeniopus*	BHK1, v1
	BTR139707	Almirante, Panama	1965	Unknown	Unknown
IF	78V3531	Brazil	1978	Mosquito	dec1, sm3, v1
II	Fe-37c	Florida	1963	*Culex* (*Melanoconion*) sp.	sm4, v6
	Fe5-47et	Florida	1965	*Aedes taeniorhynchus*	sm2
IIIA	BeAn8	Near Belem, Brazil	12-27-54	Monkey	p8
IIIB	CaAn410d	Tonate, French Guiana	1-16-73	Bird	sm3, v1
	CM4-146	Colorado	1974	*Oeciacus vicarius*	v6, cec2
IIIC	71D1252	Iquitos, Peru	1971	Mosquito	sm1
IV	BeAr35645	Near Belem, Brazil	12-61	*Anopheles nimbus*	sm4
V	CaAr19007	French Guiana	1974	Mosquito	sm4, cec1
VI	Ag80-663	Argentina	1980	Mosquito	p4, sm2

[a] St., state; Dept., department.
[b] Expressed as year, month-year, or month-day-year.
[c] Indicated as cell type (BHK, baby hamster kidney cell; C6/36, mosquito cell; cec, chicken embryo cell; dec, duck embryo cell; gp, guinea pig; h, horse; p, unknown passage; sm, suckling mouse; v, Vero cell) followed by number of passages.

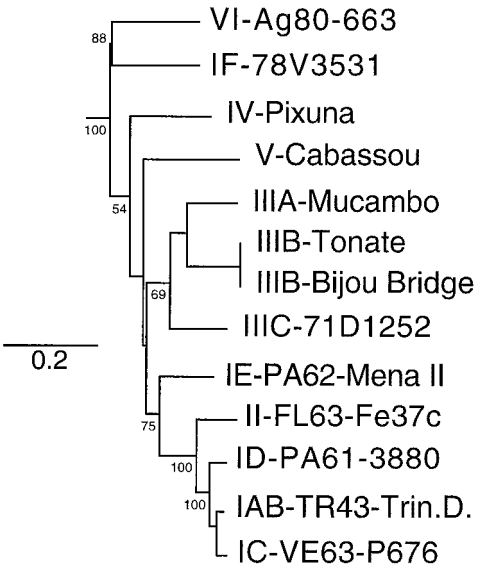

FIG. 1. Phylogenetic tree of prototype VEE complex viruses generated from 817-nucleotide sequences of E3-E2 PCR products (857-bp products excluding the primer sequences), using the PAUP parsimony algorithm and transition/transversion 1:4 weighting and drawn by using the FITCH distance program to correct branch lengths. Numbers indicate PAUP bootstrap values for groups to the right. The bar represents nucleotide sequence divergence.

II, and III viruses (bootstrap values of 91 to 100) representing monophyletic groups descended from a common ancestor. Again, the closest relatives of the IAB and IC epidemic/epizootic strains were the subtype ID and II (Everglades) viruses (Fig. 2).

Within the IAB/IC/ID/II clade, four distinct genotypes, differing by 5 to 25% in their nucleotide sequences and 1 to 12% in their deduced amino acid sequences, were delineated: (i) four ID isolates from Tumaco, in southwestern Colombia, and coastal Ecuador; (ii) Everglades viruses from southern Florida; (iii) ID strains from Panama and Iquitos, Peru (the probable recent introduction of this Panamanian genotype into Peru is discussed in detail elsewhere [22]); and (iv) a large group of ID viruses from Colombia, Venezuela, and Peru, which also included all epidemic/epizootic strains examined. As in previous analyses, the IAB strains comprised a single clade and were very closely related, differing by a maximum of only 0.7% in their nucleotide sequences. However, the IC viruses fell into at least two distinct groups; the 1962–1964 and 1995 IC isolates from northern Colombia and Venezuela grouped together as in previous analyses (42), while the 1992–1993 Venezuelan isolates grouped with enzootic ID strains from the same region of northern Venezuela and nearby Colombia. Two ID 1981 isolates from Sinamaica, in northwestern Venezuela near the Guajira Peninsula, were even more closely related to these 1992–1993 IC strains than strain 83U434 (CO83) reported previously (26); these Sinamaica strains differed from the IC viruses by only 11 nucleotides (1.3%) in the genome region that we sequenced. These results provide even stronger support for the hypothesis that the 1992–1993 outbreak in Trujillo and Zulia states of Venezuela resulted from the recent emergence of sympatric, enzootic ID viruses. The Colombia 1961 IC strain (V178) was not securely placed in our tree, and its epidemiological history is unclear. It may represent the ancestor of viruses causing the 1962–64 and 1995 outbreaks which arose in central Colombia, or a separate and more limited IC emergence event.

While IC strains from the 1992–1993 outbreak grouped closely with sympatric, enzootic ID viruses, the remaining epidemic/epizootic groups (IAB and 1962–64/1995 IC) had enzootic sister groups that were more distantly related, as indicated by the relatively long branches separating them from the remainder of the Colombia-Venezuela-Peru ID group. These long branches may reflect a period of rapid genetic change accompanying an enzootic to epidemic transition, or the lack of closely related enzootic viruses in our collection due to incomplete sampling or extinction of parental lineages. The lack of rapid change indicated by the short branches separating the 1993 IC isolates from enzootic ID strains closely related to the predicted progenitor (Fig. 2) is consistent with the incomplete sampling or extinction hypotheses.

E2 sequences. Because changes in the E2 envelope glycoprotein have been implicated in mouse attenuation of the TC-83 vaccine strain (13) and other laboratory mutants derived from the Trinidad donkey IAB strain (3), we sequenced an additional PCR fragment, including nucleotides 9047 to 10087, for representatives of all major groups identified in our initial phylogenetic analysis (Fig. 2) to generate complete PE2-6K sequences. Phylogenetic analyses using these sequences of 1677 nucleotides generated trees (not shown) completely consistent with those from the shorter 817-nucleotide sequences. Potential E2 determinants of natural equine virulence were investigated and compared with mouse virulence determinants by comparing E2 amino acid sequences of subtype IAB/IC/ID/II viruses with those of the laboratory-attenuated strains (3, 13) (Fig. 3). Most informative amino acid differences (those with two or more different residues found in two or more different strains) correlated with phylogenetic relationships rather than phenotypic characteristics and are therefore probably not responsible for differences in virulence or serotype.

Analysis of E2 amino acid determinants implicated previously in laboratory attenuation for mice indicated that they are not involved as natural determinants of equine virulence. E2 amino acid 120 is the major structural determinant of virulence differing between the TC-83 vaccine strain and its virulent IAB Trinidad donkey parent (13). However, E2-120 does not appear to play a role in natural virulence or VEE emergence, since all VEE virus strains that we sequenced, including virulent IC and enzootic ID isolates, have T-120 like Trinidad donkey (Fig. 3). Likewise, E2 amino acids 76 and 209, which differ between Trinidad donkey and attenuated mutants generated by selection for rapid cell penetration (3), did not vary among the natural isolates that we sequenced. The complete E2 amino acid sequences revealed no common mutations that unite all epidemic/epizootic (IAB/IC) or even all variety IC strains. Both IC genotypes (1962–1964/1995 and 1992–1993) had mutations of E2 amino acid 117 (glycine for the 1961–1995 group and asparagine for the 1992–1993 strains) with respect to IAB and most ID isolates. Asparagine-117 was also found in all other 1992–1993 epizootic/epidemic isolates, including SH5, which is intermediate between ID and IC in its reactivity with monoclonal antibodies in ELISA (26). However, the closest relatives of the 1992–1993 IC and ID/IC isolates, ID strains 66457 and 66637, also had the asparagine at position 117. To confirm their serotype classification, which had been determined previously by using hemagglutination inhibition assays (34), strains 33571 and 66457 were reanalyzed by using an ELISA and monoclonal VEE virus subtype-specific antibodies as described previously (24, 26). Both of these strains reacted like subtype ID viruses, indicating that amino acid 117 alone is not solely responsible for the IC phenotype.

Amino acids 182 to 207 comprise four overlapping domains

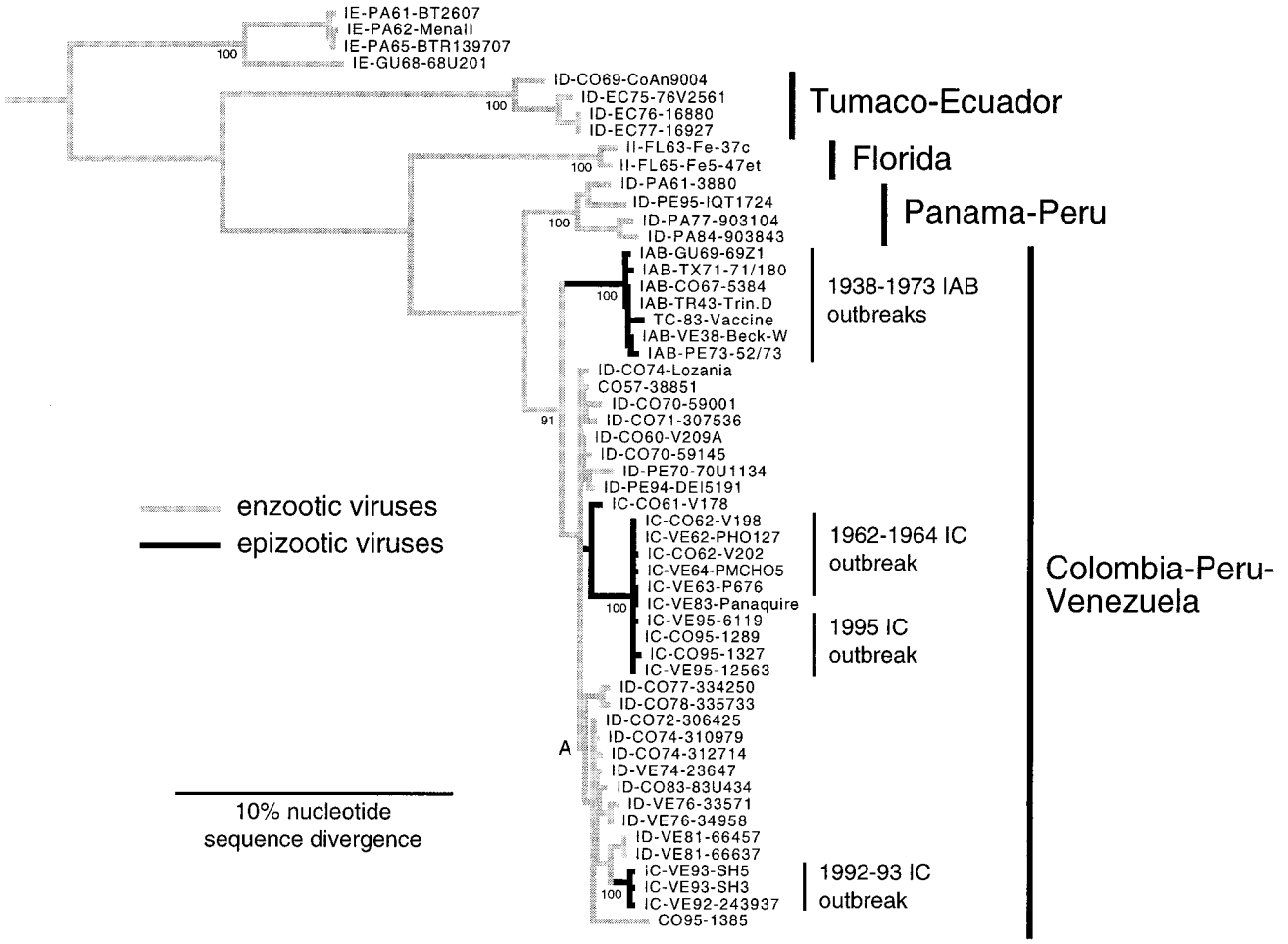

FIG. 2. Detailed phylogenetic tree of subtype IAB, IC, ID, IE, and II VEE viruses generated from 817-nucleotide sequences of E3-E2 PCR products (857-bp products excluding the primer sequences) with the PAUP parsimony program, using unordered characters. Numbers indicate bootstrap values for groups to the right. Letter A indicates hypothetical ancestral sequence used for estimation of evolutionary rates of enzootic VEE viruses. Branch shading indicates the most parsimonious reconstruction of ancestral epidemiological traits for VEE virus lineages. The bar indicates horizontal distance equating to 10% nucleotide sequence divergence.

containing important neutralization epitopes (10, 27, 28). Because virus neutralization assays have been used to designate serotype, we looked for the presence of changes in this region specific for distinct serotypes. Two amino acid changes were identified, each specific to one of the IC genotypes (arginine-193 for the 1992–1993 strains and lysine-201 for the 1962–1995 strains). The lack of any E2 mutations common to both IC genotypes suggests that different mutations or combinations can lead to the IC phenotype and/or equine virulence.

The correlation between phenotype or virulence and potential N-linked glycosylation sites present in the E2 gene was also examined. While all IAB strains contain three such sites (at amino acids 212 to 214, 291 to 293, and 318 to 320), only the latter two sites are present in most IC, ID, and IE isolates examined here; the substitution of an alanine for threonine at amino acid 214 destroys the first E2 glycosylation site. Interestingly, while the Asn-X-Thr site at E2 positions 212 to 214 is retained in subtype II viruses, the second potential site at 291 to 293 is ablated. Again, no consistent pattern emerged to distinguish epidemic/epizootic viruses from enzootic strains.

Rates of enzootic evolution. One hypothetical explanation for the repeated emergence of epidemic/epizootic VEE viruses in the IAB and 1963–1995 IC groups is the maintenance of these strains in enzootic transmission cycles not yet identified. The recent finding of a 1983 variety IC isolate from mosquitoes in Panaquire, Venezuela, isolated during a 19-year hiatus of epidemic/epizootic VEE activity, supports this hypothesis (42). To test this hypothesis further, we estimated the evolutionary rate accompanying enzootic VEE virus transmission in South America by analyzing mutations occurring in a closely related group of viruses sampled over a relatively long time period as described previously (2, 37). A group of enzootic viruses from Colombia and Venezuela, descended from hypothetical ancestor A (Fig. 2, node A) and including strains isolated from 1972 to 1995, yielded an estimated evolutionary rate of 7×10^{-4} substitutions per nucleotide per year (regression coefficient = 0.91). This estimate yielded expected numbers of mutations of 20 from 1938 to 1973 for the IAB viruses and 19 from 1962 to 1995 for the IC viruses. The lack of any mutations in the consensus sequences of the IAB viruses isolated from 1938 to 1973 or IC viruses isolated from 1962 to 1995 indicates that if these viruses were circulating during these time periods, they had an evolutionary rate of less than 3.5×10^{-5}, roughly 20-fold lower than the rates for the ID viruses described above and 10-fold lower than the rates for other alphaviruses circulating in the New World tropics (37, 40). These data fail to

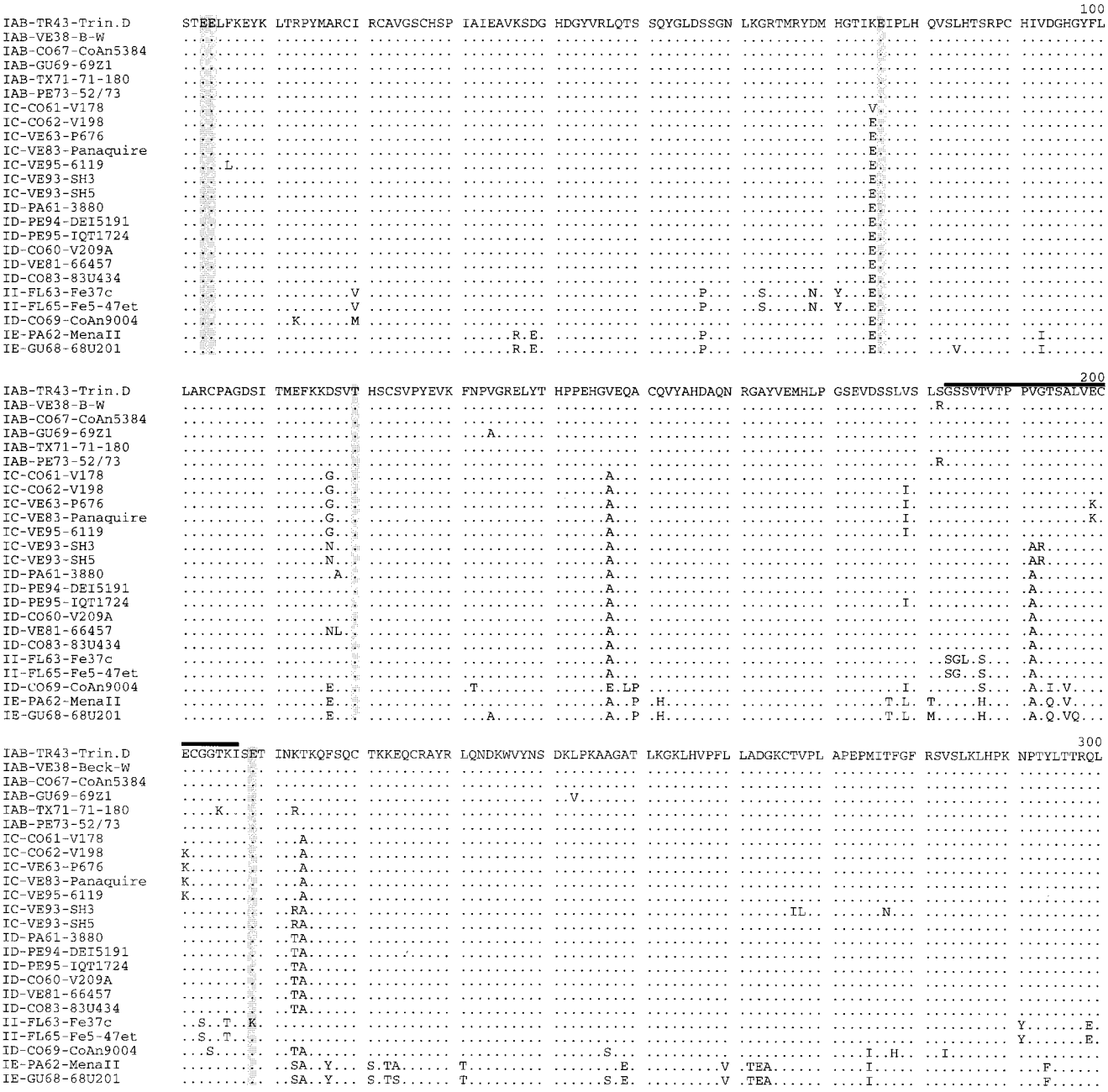

FIG. 3. Aligned E2 amino acid sequences for representative lineages from the phylogenetic tree depicted in Fig. 2. Shaded amino acids are those shown previously to contribute to mouse attenuation of the Trinidad donkey strain (3, 13). Solid horizontal bar shows locations (positions 182 to 207) of epitopes shown previously to elicit neutralizing antibodies (10, 27, 28).

support the cryptic maintenance (enzootic-like) transmission hypothesis for maintenance of IAB and IC viruses.

DISCUSSION

Emergence of epidemic/epizootic VEE. Results of our analyses support the hypothesis that epidemic/epizootic VEE viruses are generated by mutation of enzootic, subtype ID viruses in northern South America. Our results suggest that different combinations of critical mutations occur periodically in ID viruses, leading to antigenic changes and enhanced equine viremia. This high-titered viremia results in the potential for efficient transmission from horse to horse by a variety of mammalophilic mosquitoes of only moderate susceptibility. Under appropriate ecological conditions, including an adequate supply of susceptible equines and mosquitoes, epizootic transmission ensues. Humans may become infected tangentially when infected mosquitoes bite people (11, 36). However, the moderate viremia titers measured in infected people during several outbreaks (1, 5, 18, 42) may exceed infection thresholds of some epizootic/epidemic vector species (32), allowing for epidemic transmission in the absence of susceptible equines. This possibility deserves further attention, as equines are increasingly replaced by motor vehicles in many parts of

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IAB-TR43-Trin.D      ADEPHYTHEL ISEPAVRNFT VTEKGWEFVW GNHPPKRFWA QETAPGNPHG LPHEVITHYY HRYPMSTILG LSICAAIATV SVAASTWLFC RSRVACLTPY
IAB-VE38-Beck-W      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
IAB-CO67-CoAn5384    .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
IAB-GU69-69Z1        .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
IAB-TX71-71-180      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
IAB-PE73-52/73       .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
IC-CO61-V178         .......... .......... .......... .......... .......... .......... .......... .......V.. .......... K...S.....
IC-CO62-V198         .......... .......... .......... .......... .......... .......... .......... .......V.. .......... K...S.....
IC-VE63-P676         .......... .......... .......... .......... .......... .......... .......... .......V.. .......... K...S.....
IC-VE83-Panaquire    .......... .......... .......... .......... .......... .......... .......... .......V.. .......... K...S.....
IC-VE95-6119         .......... .......... .......... .......... .......... .......... .......... .......V.. .......... K...S.....
IC-VE93-SH3          .......... .......... .......... .......... .......... .......... .......... .......V.. .......... K...S.....
IC-VE93-SH5          .......... .......... .......... .......... .......... .......... .......... .......V.. .......... K...S.....
ID-PA61-3880         .......... ....V....S .......... .......... .......... .......... .......... .......V.. .I......L. K...S.....
ID-PE94-DEI5191      .......... .......... .......... .......... .......... .......... .......... .......VI. .......... K...S.....
ID-PE95-IQT1724      T......... .........S .......... .......... .......... .......... .......... .......V.. .I......L. K...S.....
ID-CO60-V209A        .......... .......... .......... .......... .......... .......... .......... .......V.. .......... K...S.....
ID-VE81-66457        .......... .......... .......... .......... .......... .......... .......... .......V.. .......... K...S.....
ID-CO83-83U434       .......... .......... I......... .......... .......... .......... .......... .......V.. .......... K...S.....
II-FL63-Fe37c        .......... ....S....S ..A....... .......... .......... ......V... ........T. .......VA. .I......L. ...AS.....
II-FL65-Fe5-47et     .......... ....S....S ..A....... .......... .......... ......V... ........T. .......V.. .I......L. ...AS.....
ID-CO69-CoAn9004     DG..N..... ..Q....... .......... .....Q.... .......... W......... ........V. ......VVVT .L......L. K...S.....
IE-PA62-MenaII       DG..A..... .TN.V....S .......... .....Q.Y.S .......... .......... .......... .......V.T .I...V.... K..IS.....
IE-GU68-68U201       DG..A..... .TH.V....S .......... .....Q.Y.S .......... .......... .......... .......V.T .I...V.... K..IS.....

                                            423
IAB-TR43-Trin.D      RLTPNARIPF CLAVLCCART ARA
IAB-VE38-Beck-W      .......... .......... ...
IAB-CO67-CoAn5384    .......... .......... ...
IAB-GU69-69Z1        .......... .......... ...
IAB-TX71-71-180      .......... .......... ...
IAB-PE73-52/73       .......... .......... ...
IC-CO61-V178         .......M.L .......... ...
IC-CO62-V198         .......M.L .......... ...
IC-VE63-P676         .......M.L .......... ...
IC-VE83-Panaquire    .......M.L .......... ...
IC-VE95-6119         .......M.L .......... ...
IC-VE93-SH3          .......M.L .......... ...
IC-VE93-SH5          .......M.L .......... ...
ID-PA61-3880         .......M.L .......... ...
ID-PE94-DEI5191      .......M.L .......... ...
ID-PE95-IQT1724      .......M.L .......... ...
ID-CO60-V209A        .......M.L .......... ...
ID-VE81-66457        .......M.L .......... ...
ID-CO83-83U434       .......M.L .......... ...
II-FL63-Fe37c        ......KM.L .........S ...
II-FL65-Fe5-47et     ......KM.L .........S ...
ID-CO69-CoAn9004     .....V.L.L .......... ...
IE-PA62-MenaII       .......M.L .......... ...
IE-GU68-68U201       ......SM.L .......... .K.
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FIG. 3—*Continued.*

Latin America. Theoretically, epidemic transmission could select for VEE viruses producing higher human viremia, which could result in increased virulence and higher mortality rates. Epidemic transmission in the absence of equines could also result in large urban outbreaks and increased risk of epidemic introduction into previously unaffected regions by infected air travelers.

The mutations responsible for VEE emergence may occur in enzootic hosts such as rodents or *Culex* (*Melanoconion*) vectors or in epizootic equine or mosquito hosts. Mutations occurring within enzootic foci could undergo selection when epizootic vectors bite infected hosts within enzootic foci, followed by transmission to nearby equines, or when enzootic vectors transmit to equines near enzootic forest or swamp habitats. The greater replication in equines of epizootic IAB/IC strains than of ID enzootic isolates (35) suggests that equines are a likely site of selection. However, infection experiments are needed to evaluate the possibility that epizootic vector species offer an alternative selection site.

The lack of correlation in E2 sequence differences between equine-virulent IAB/IC and avirulent ID/IE/II strains and E2 amino acid differences that are determinants of mouse attenuation (3, 13) is not altogether surprising. VEE virus virulence for laboratory rodents does not always correlate with that for equines. For example, hamsters are uniformly susceptible and die after infection with small doses of all subtypes except IV (Pixuna). English short-hair guinea pigs survive infection with most enzootic subtypes, including IE. However, some strains of ID viruses, including 3880 and V209A, produce lethal infections (29). When tested in adult (14) and immature (19) Swiss NIH mice, strain 3880 is even more virulent than prototype, epizootic IAB and IC strains. Until a better animal model is identified, experimental infections of equines will be required for studies of epizootic VEE determinants.

Our phylogenetic analyses indicate at least three epidemic/epizootic VEE emergence events from enzootic ID-like viruses. However, these three emergences are all associated with only one of four ID-like genotypes that we identified. The lack of VEE emergence from genotypes circulating in southwestern Colombia-coastal Ecuador, Florida, or Panama-Peru, all of which are inhabited by susceptible equines, has several possible explanations, including (i) a chance lack of the simultaneous occurrence of appropriate mutations and ecological conditions required for VEE emergence or (ii) genetic differences in these other ID-like viruses, with respect to the Colombia-Peru-Venezuela ID genotype, requiring more mutations to produce equine viremia sufficient for epizootic emergence. More detailed analyses of the mutations responsible for the 1992 epizootic transition, revealed by complete genomic sequences of the 1992–1993 IC isolates and closely related, sympatric enzootic ID strains, may identify some mutations critical to emergence. This information, combined with mutagenesis of infectious cDNA clones derived from all four ID-like genotypes, could be used to evaluate these hypotheses and their obvious public health implications.

Reemergence of IAB and IC viruses. While the 1992–1993 outbreak apparently resulted from the recent emergence from a sympatric ID enzootic progenitor strain (26) (Fig. 2), the

remaining epidemic/epizootic IAB and IC groups each include isolates spanning a 31- to 35-year time period. The 1983 Panaquire IC isolate, identical in sequence to a 1963 epidemic/epizootic strain from the same region of northern Venezuela, may represent an enzootic-like serotype IC transmission cycle from which reemergence occurred in 1995. This isolate may be the descendent of an epizootic/epidemic IC strain from the 1962–1964 outbreak which became established in a maintenance transmission cycle there. An alternate hypothesis is that the Panaquire isolate is very closely related to the ancestor of both 1962–1964 and 1995 outbreaks. Field studies are currently under way to confirm the existence of such an enzootic, subtype IC cycle in Venezuela. However, our evolutionary studies of enzootic VEE viruses indicate that if IC viruses are continuously circulating in Venezuela, their evolutionary rate must be an order of magnitude lower than those of other alphaviruses that we have examined.

An alternative hypothesis for interepizootic maintenance of IAB or IC viruses is some kind of latent infection with little or no genome replication, resulting in genetic stasis. However, the life span of equines and enzootic rodent hosts, as well as mosquito vectors, is less than the 31-year time interval between the 1962–1964 and 1995 variety IC outbreaks. The most likely candidate for latent VEE virus maintenance would therefore be longer-lived human hosts. However, no evidence of persistent human infection has been reported.

Finally, the genetic stasis that we observed for some epidemic/epizootic strains does support the hypothesis that later IAB outbreaks, such as the 1973 Peru epizootic, resulted from the use of incompletely inactivated vaccine preparations made from the Trinidad donkey, Beck-Wycoff, or other early isolates (11, 17, 36). For example, this hypothesis is supported by the grouping of the 1938 Beck-Wycoff isolate with one isolated in Peru during 1973 (Fig. 2). However, vaccination cannot be used to explain the more recent IC outbreaks, because to our knowledge, variety IC viruses have not been used for vaccine production. More detailed genetic studies examining a larger portion of the viral genome, to delineate mutations critical to the epizootic phenotype, combined with field investigations to examine the possibility of continuous transmission of IC viruses in Venezuela, may elucidate interepizootic maintenance mechanisms for these VEE viruses.

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