JOURNAL OF VIROLOGY, Jan. 2003, p. 1204–1210 Vol. 77, No. 2
0022-538X/03/$08.00+0 DOI: 10.1128/JVI.77.2.1204–1210.2003

Glycosaminoglycan Binding Properties of Natural Venezuelan Equine Encephalitis Virus Isolates

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Received 1 July 2002/Accepted 7 October 2002

Equine-virulent, epidemic/epizootic strains of Venezuelan equine encephalitis (VEE) virus (VEEV) arise via mutation of progenitor enzootic strains that replicate poorly in equines. Sequencing studies have implicated positively charged amino acids on the surface of the E2 envelope glycoprotein in the acquisition of equine virulence and viremia potential, suggesting that changes in binding to cell surface glycosaminoglycans (GAGs) may mediate VEE emergence. Therefore, we evaluated the binding of natural enzootic and epizootic VEEV isolates to Chinese hamster ovary (CHO) cells expressing normal, high levels of GAGs as well as to mutant CHO cells lacking GAG expression. Binding to GAGs was not consistently associated with the epizootic phenotype, and cell culture passages resulted in increased GAG binding. The low levels of GAG binding exhibited by some low-passage, equine-virulent subtype IC VEEV strains indicate that the positive-charge E2 mutations implicated in VEE subtype IC emergence are not artifacts of laboratory passage and suggest that GAG binding does not play a major role in mediating VEE emergence. The increased GAG binding exhibited by VEEV strain CPA201 from the 1993 Mexican epizootic, when compared to that of closely related enzootic subtype IE strains, was shown to result from a Glu-to-Lys mutation at position 117 of the E2 envelope glycoprotein.

Venezuelan equine encephalitis (VEE) *virus* (VEEV) is an *Alphavirus* in the family *Togaviridae*, a group of enveloped, single-stranded, positive-sense RNA viruses (9a, 27). The genome encodes four nonstructural proteins (nsP1 to nsP4) involved in genome replication and three major structural proteins (capsid and E2 and E1 envelope glycoproteins) which are expressed from a 26S subgenomic message colinear with the 3′ one-third of the genomic RNA. The E2 envelope protein forms spikes on the surface of the virion that interact with cellular receptors for viral entry and include the major antigenic determinants that define VEEV subtypes (9a).

VEEV has caused periodic epidemic and equine epizootic outbreaks in the Americas dating from the early 20th century. The most recent outbreaks occurred in 1992 and 1993 in western Venezuela (19), in 1993 and 1996 in southern Mexico (16), and in 1995 in northern Venezuela and Colombia (26). With the exception of the Mexican outbreaks, all of the known VEE epizootics have involved VEEV in two antigenic subtypes, IAB and IC. Phylogenetic studies indicate that these viruses evolve periodically from only one of five genotypes of enzootic serotype ID viruses (17, 19, 25). These progenitors of the epizootic viruses circulate continuously or semicontinuously in humid tropical forest habitats in western Venezuela, eastern and central Colombia, and northern Peru (1, 15, 21). Unlike the epizootic IAB and IC viruses that exploit equines as amplification hosts by generating high-titered viremia as required for infection of mosquito vectors, the enzootic ID strains are generally equine avirulent and generate little or no viremia (25). Some epizootic VEEV strains also infect certain epizootic mosquito vectors more efficiently than enzootic strains, and the efficient infection phenotype is mediated by the E2 envelope glycoprotein (5).

Using phylogenetic approaches, we identified seven amino acid changes in the VEEV genome that represent putative determinants of emergence of the 1992 to 1993 epizootic/epidemic serotype IC strains from enzootic ID viruses circulating sympatrically in western Venezuela (24). E2 envelope glycoprotein sequences from VEEV strains representing all major outbreaks indicate that VEE emergence is associated with mutations that increase the charge on the surface of the E2 envelope glycoprotein (3). These mutations result in a small-plaque phenotype that is characteristic of nearly all epizootic VEEV strains (3, 14). Reverse genetic studies are under way to determine which of these changes are responsible for the switch in equine virulence and viremia that leads to outbreaks.

Cell culture passage of alphaviruses, including VEEV (2) and Sindbis virus (7, 13), results in selection for binding to heparan sulfate (HS), and this selection is accompanied by mutations that increase the positive charge of E2 and the small-plaque phenotype characteristic of natural, epizootic VEEV strains (3). Paradoxically, the artificial, cell culture passage-associated HS binding mutations attenuate VEEV (2) and Sindbis virus (7, 13) for mice, while natural VEEV mutations that increase the E2 positive charge are associated with equine virulence. These data suggest that changes in binding to cell surface glycosaminoglycans (GAGs) mediate VEE emergence via altered binding to certain equine cells.

We evaluated the hypothesis that GAG binding mediates VEE emergence by assessing the binding of natural enzootic and epizootic VEEV isolates to Chinese hamster ovary (CHO)

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TABLE 1. Alphavirus strains studied

Strain[a]	Subtype	Epidemiological type[b]	Plaque size (14, 24)	Date of isolation	Place of isolation	Host	Passage history[c]
EgAr339 (Sindbis)	NA[d]	NA	NA	1953	Egypt	Mosquito	V6, BHK1
Trinidad donkey (TRD)	IAB	Epizootic	Small	1943	Trinidad	Donkey	GP1, V6, BHK1
69Z1	IAB	Epizootic	Small	1969	La Avellana, Guatemala	Human	SM2
V198	IC	Epizootic	Small	1962	Guajira, Colombia	Human	SM1, V1
V202	IC	Epizootic	Small	1962	Guajira, Colombia	Human	SM1, V1
P676	IC	Epizootic	Small	1963	Miranda State, Venezuela	Mosquito	SM1, V1
243937	IC	Epizootic	Small	18 December 1992	Trujillo State, Venezuela	Horse	SM2
SH3	IC	Epizootic	Small	9 January 1993	Trujillo State, Venezuela	Human	V1
125573	IC	Epizootic	Small	June 1993	Zulia State, Venezuela	Human	V1
6119	IC	Epizootic	Small	31 May 1995	Falcon State, Venezuela	Human	V1
3908	IC	Epizootic	Small	September 1995	Zulia State, Venezuela	Human	C6/36-1
V209A	ID	Enzootic	Large	1960	Santander, Colombia	Mouse[e]	SM2, V2
66637	ID	Enzootic	Large	19 November 1981	Zulia State, Venezuela	Hamster[e]	SM1, V1
66457	ID	Enzootic	Large	11 November 1981	Zulia State, Venezuela	Hamster	SM1, V1
83U434	ID	Enzootic	Large	June 1983	N. Santander, Colombia	Hamster	CEC1, V1
ZPC738	ID	Enzootic	Large	24 September 1997	Zulia State, Venezuela	Hamster	BHK1
68U201	IE	Enzootic	Large	1968	La Avellana, Guatemala	Hamster	SM1, BHK2
CPA201	IE	Epizootic	Small	29 June 1993	Chiapas State, Mexico	Horse	SM1, RK1

[a] All are strains of VEEV except strain EgAr339, which is a strain of Sindbis virus.
[b] Epizootic strains were isolated during equine outbreaks; enzootic strains were isolated from sylvatic habitats and are not associated with outbreaks.
[c] CEC, chick embryo cells; GP, guinea pig; RK, rabbit kidney; SM, suckling mouse; V, Vero cells. Numerals represent numbers of passages.
[d] NA, subtypes, plaque sizes, and epidemiological conditions are not applicable for Sindbis virus.
[e] Mice and hamsters were sentinels placed into enzootic (sylvatic) VEEV habitats.

cells expressing normal, high levels of GAGs, including HS, as well as of mutant CHO cells lacking GAG expression. Our results indicated that GAG binding is not strongly associated with the natural, epizootic VEEV phenotype.

MATERIALS AND METHODS

Virus strains. The enzootic and epizootic VEEV strains we used are listed in Table 1. These included the 1943 epizootic subtype IAB strain Trinidad donkey (one guinea pig passage, six Vero cell passages, and one BHK cell passage before cloning), rescued from the infectious cDNA clone PVE/IC-109 as described previously by Kinney et al. (11), and the 1969 IAB strain 69Z1. Seven subtype IC epizootic strains included two from the 1962 to 1964 Venezuelan epizootic (6), V-198 and V-202 (4); three from the 1992 to 1993 outbreak, 243937, 125573, and SH3 (19); and two from the 1995 outbreak, 6119 and 3908 (26). For strain 3908, wild-type virus after the first C6/36 mosquito cell passage, as well as virus rescued from the infectious cDNA clone p3908.acb (generated from RNA obtained from the first C6/36 cell passage) (5), was used. We also analyzed equine epizootic strain CPA201 from the 1993 Chiapas, Mexico, outbreak (16). Four enzootic subtype ID strains from Colombia and Venezuela were included, 209A, 66637, 66457, and ZPC738, as well as enzootic subtype IE strain 68U201 derived from an infectious clone, pIE.AA (clone produced from RNA generated after one suckling mouse passage and two BHK passages) (17). Sindbis virus strain EgAr339 (acquired from the University of Texas Medical Branch World Reference Center for Arboviruses) was used as a positive control for GAG binding studies. The passage histories and other pertinent data are listed in Table 1.

Mutagenesis of the infectious cDNA clone pIE.AA. The infectious cDNA clone pIE.AA, derived from the Guatemalan enzootic subtype IE strain 68U201, has been described previously (17). To test the effect of the Glu-to-Lys mutation that accompanied the 1993 VEE emergence in Mexico (3), pIE.AA was mutagenized to place the Lys residue in the enzootic backbone. Generation of a PCR amplicon from pIE.AA cDNA genomic positions 8875 to 9821 was performed with the mutagenesis primers Lys-IE-8875(+) (5′-GCCGAATTCCCTGCAGGAGACT CCATAACTATGGAATTTAAGAAAAAATCAGTCACCC-3′) (encoding the Glu-to-Lys substitution) and IE-9821(−) (5′-CAGCAGAATTCGGCTAAGCA CAGAGGCATGCTGGCATTTGGAGTC-3′). Both the pIE.AA vector and amplicon were digested with *Blp*I and *Sse*8387I, and the digestion products were gel purified and ligated. Clones positive for insertion were sequenced to confirm the incorporation of the Lys residue. Infectious virus was rescued from the plasmid as previously described (17).

Cell lines and culture reagents. Chinese hamster ovary (CHO-K1) cells (CCL-61) and their GAG-deficient CHO derivative pgsA-745 (CRL-2242) were purchased from the American Type Culture Collection (Bethesda, Md.) and grown in Ham's F-12 medium supplemented with 10% fetal bovine serum (FBS) and 50

μg of gentamicin/ml. Baby hamster kidney (BHK-21) and Vero monkey kidney cells were propagated in Eagle's minimal essential medium (MEM) supplemented with 5% FBS. *Aedes albopictus* (C6/36) mosquito cells were grown in MEM supplemented with 10% FBS and 1% nonessential essential amino acids. Heparin and heparinase I were obtained from Sigma (St. Louis, Mo.).

Labeling of VEEV with [^{35}S]methionine and virus purification. [^{35}S]methionine-labeled viruses were prepared by infection of BHK-21 cells with stock viruses at a multiplicity of infection of approximately 10. Following 4 h of incubation at 37°C, the medium was replaced with methionine-free MEM for 3 h. [^{35}S]methionine (Amersham Biosciences, Piscataway, N.J.) was added to the medium at 15 μCi/ml. Supernatant fluid was collected when cytopathic effects were evident and clarified by centrifugation at 3,000 × *g* for 10 min. Virus was precipitated with polyethylene glycol 8000 and NaCl at final concentrations of 7 and 2.3% (wt/vol), respectively, and pelleted at 6,000 × *g* for 30 min. The viruses were purified on continuous 20 to 70% (wt/vol) sucrose gradients in TEN buffer (0.05 M Tris-HCl [pH 7.2], 0.1 M NaCl, 0.001 M EDTA) and pelleted at 270,000 × *g* for 90 min.

Cell-binding assays. CHO cells were plated at 4×10^5 cells per cm^2 and used the following day after incubation at 37°C. The cells were rinsed twice with ice-cold binding buffer (phosphate-buffered saline [PBS] with 0.5 mM $MgCl_2$ and 1 mM $CaCl_2$) with 0.5% bovine serum albumin. About 10^4 cpm of ^{35}S-labeled virus was added to each well in 150 μl of binding buffer, and plates were rocked at 4°C for 1 to 3 h. Following incubation, unbound virus was removed and the cells were rinsed twice with ice-cold binding buffer. The cells were then lysed in 1% sodium dodecyl sulfate, and counts per minute were assayed by liquid scintillation counting.

Heparinase I digestion of CHO-K1 cells. Prior to virus binding, cells in 12-well plates were treated with 10 U of heparinase I (Sigma) in PBS with 0.1% bovine serum albumin or mock digested for 1 h at 37°C.

Binding competition experiments with heparin. Heparin, an HS-like molecule, was used for binding competition experiments. Virus was diluted to approximately 100 PFU in 200 μl of binding buffer and preincubated with various concentrations of heparin for 30 min at 4°C. The mixture was then added to Vero cells for 2 h at 4°C and washed two times with PBS. Monolayers were overlaid with 4 ml of MEM containing 2% FBS and 0.4% agarose. After 48 h, the cells were fixed in 10% formaldehyde and stained with 0.5% crystal violet for plaque counts.

RT-PCR amplification and sequencing. The complete E2 and E1 envelope glycoprotein genes of selected virus strains were amplified by reverse transcription-PCR (RT-PCR) as described previously (3). PCR amplicons were sequenced directly with an Applied Biosystems (Foster City, Calif.) model 3100 automated sequencer and the BigDye sequencing kit according to the manufacturer's protocols.

RESULTS

South American VEEV strains. Emergence of epizootic VEEV has been associated with mutations that increase the positive charge on the E2 envelope (3). Because similar mutations are known to increase the binding of Sindbis virus (7, 13) and VEEV (2) to HS, we assessed the binding of enzootic and epizootic VEEV strains using CHO cells. The relative binding affinities were compared for wild-type CHO cells versus mutant pgsA-745 cells that are defective in GAG synthesis. Initial studies focused on the most closely related enzootic and epizootic strains, including comparison of two VEEV subtype IC epizootic strains from the 1992 outbreak, 243937 and SH3, with the closely related enzootic subtype ID strains 66637 and ZPC738 (24). These viruses demonstrated small but consistent differences in CHO cell binding. However, none bound with the same affinity as cell culture-adapted Sindbis virus (Fig. 1A). The VEEV binding differences were almost completely eliminated when binding was tested using the GAG-deficient mutant pgsA-745 cells (Fig. 1B), indicating that the binding difference resulted from differential GAG binding on the surface of the CHO cells.

The effect of heparinase on CHO cell binding was also used to determine whether HS was the target of CHO cell binding.

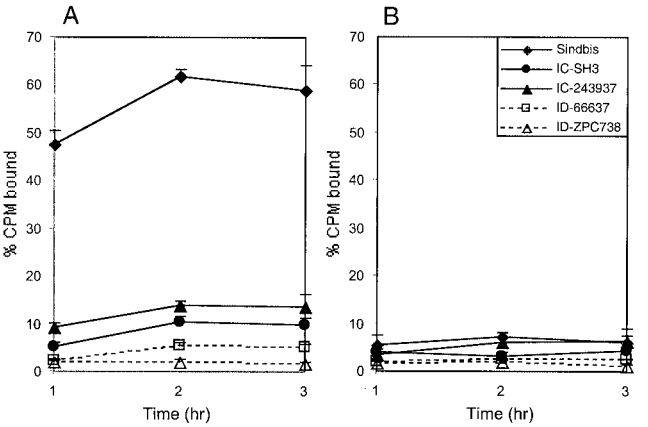

FIG. 1. Binding of purified, radiolabeled VEEV strains and Sindbis virus control to CHO cells. (A) Binding to CHO-K1 cells; (B) binding to pgsA-745 (GAG-deficient) CHO-K1 cells. Bars indicate standard deviations.

The 1992 epizootic subtype IC strain 243937 and the closely related enzootic ID strain 66637 were compared, and Sindbis virus was included as a positive control. All three alphavirus strains showed a 50 to 60% reduction in CHO cell binding after heparinase treatment (Fig. 2), indicating that some of the binding involved heparinase substrates on the cell surface. As a further test of the hypothesis that enzootic and epizootic VEEV strains bind to HS and to investigate whether the GAG involved in VEEV binding to other cells was also HS, soluble heparin was used in a Vero cell blocking assay. After incubation with concentrations of soluble heparin ranging from 0.004 to 400 U/ml for 30 min at 4°C, both VEEV strains tested (243937 and 66637) showed a reduction in PFU reaching 80 to 93% at the highest concentration (Fig. 3). These results indicated that both enzootic and epizootic VEEV also bind to GAGs on the surface of Vero cells.

These initial results indicated that both subtype IC and ID VEEV can bind to cells by attachment to HS and that epizootic subtype IC strains from the 1992 outbreak have a slightly higher affinity for HS on the cell surface than closely related, equine-avirulent enzootic subtype ID strains. To determine whether this difference extends to other epizootic and enzootic VEEV strains, we tested additional isolates for binding to CHO cells. Some epizootic IAB strains (Trinidad donkey and

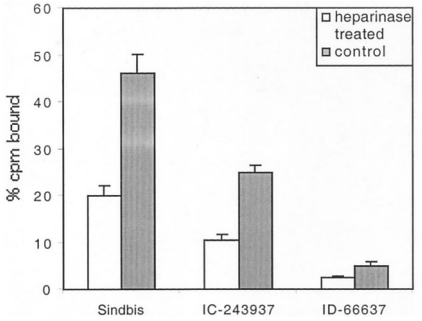

FIG. 2. Binding of VEEV strains and Sindbis control to CHO-K1 cells after pretreatment with heparinase 1 (10 U/ml). Bars indicate standard deviations.

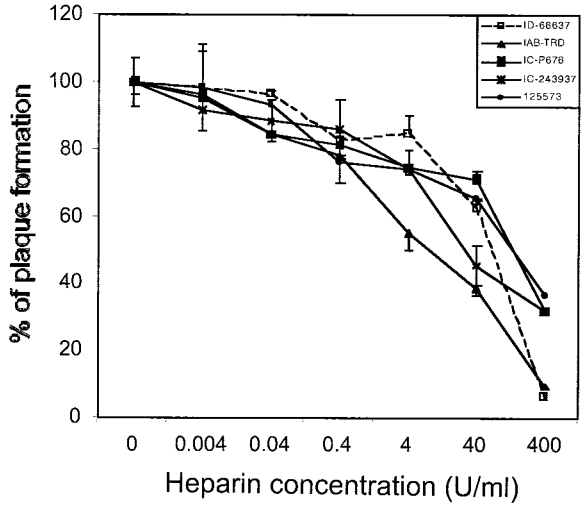

FIG. 3. Inhibition of epizootic and enzootic VEEV plaque formation by soluble heparin.

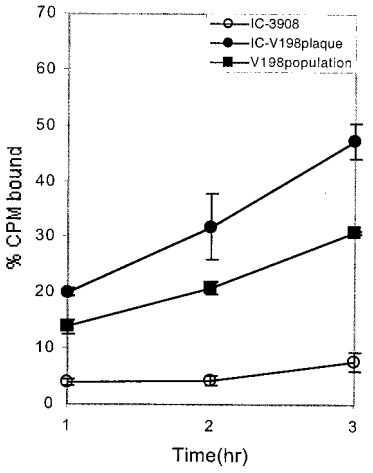

FIG. 5. Binding of plaque-purified and unpurified strain V-198, as well as virus derived from the infectious cDNA clone p3908.acb, to CHO-K1 cells. Bars indicate standard deviations.

69Z1) and IC strains (V-202 and V-198) showed higher levels of CHO cell binding than the 1992 IC strains. However, the 1995 epizootic IC strain 3908, including both wild-type virus from the first C6/36 cell passage (Fig. 4) and virus rescued from the infectious clone (Fig. 5), had binding levels comparable to those of the enzootic ID strains (Fig. 4). The CHO cell phenotype was not well correlated with plaque size (Table 1).

The difference in binding between strains V-198 and 3908 was surprising, because the structural protein consensus sequences of the stocks used to prepare radiolabeled virus were identical (4). This binding difference may have reflected a high-affinity-binding minority population within the V-198 strain. To test this hypothesis, we amplified by RT-PCR and sequenced the E2 and E1 envelope glycoprotein genes that contain determinants of HS binding. The electropherogram derived from sequencing the amplicon directly revealed a single nonsynonymous difference between the previously determined sequence of strain V-198 (GenBank accession no. U55342) and that of the population from which our radiolabeled stocks were derived. The population used for our binding studies contained a mixture of A and G nucleotides at genome position 9158 (Fig. 6), resulting in a mixture of deduced Glu and Lys amino acids at position 199 of the E2 protein. Glu is found at E2-199 in the 3908 sequences as well as the consensus V-198 sequence, while Lys has been reported previously in a high-passage strain P676 sequence but not in a lower-passage version (4). These data were consistent with the presence of a high-binding variant in the V-198 population. To confirm that the difference in binding of the V-198 mixture resulted at least in part from this mutation, we isolated a plaque from the V-198 stock that contained the Lys residue at E2 position 199 and compared the CHO cell binding of the plaque-purified virus to that of the V-198 population and to virus rescued from an infectious cDNA clone made from strain 3908 (5). The V-198 plaque-purified virus bound with higher affinity than the 3908 virus and the mixed V-198 population (Fig. 5), consistent with the role of Lys-199 in increased GAG binding.

All VEEV strains exhibited very low levels of binding to the pgsA-745 cells comparable to those of strains 66637 and 243937 (data not shown), indicating that the binding differences among the different epizootic strains involved GAGs on the CHO cells. All of the strains also showed inhibition of plaque formation by soluble heparin similar to that exhibited by strains 243937 and 66637 (data not shown). Heparin is more

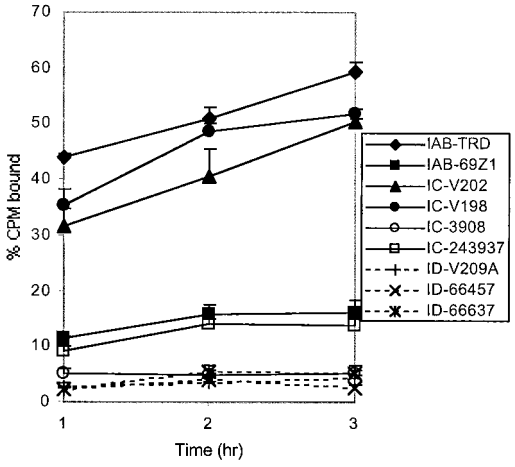

FIG. 4. Binding of additional purified, radiolabeled epizootic subtype IAB and IC strains and enzootic subtype ID VEEV strains to CHO-K1 cells. Bars indicate standard deviations.

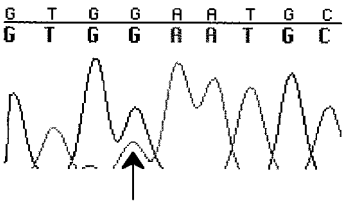

FIG. 6. Electropherogram from automated sequencer showing mixture of A (arrow) and G (consensus) peaks at genomic nucleotide position 9158.

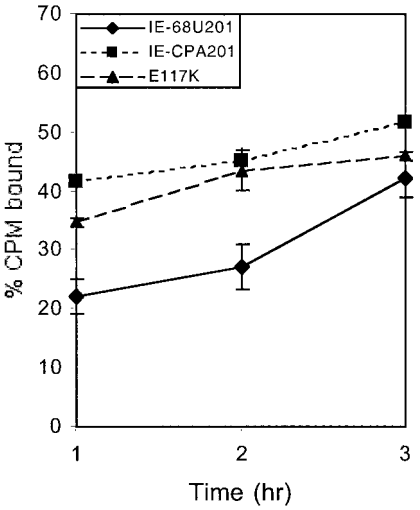

FIG. 7. Binding of purified, radiolabeled VEEV generated from the pIE.AA clone derived from enzootic subtype IE strain 68U201, the closely related Mexican epizootic strain CPA201, and the mutant containing the E2-117 Lys residue in the 68U201 backbone. Bars indicate standard deviations.

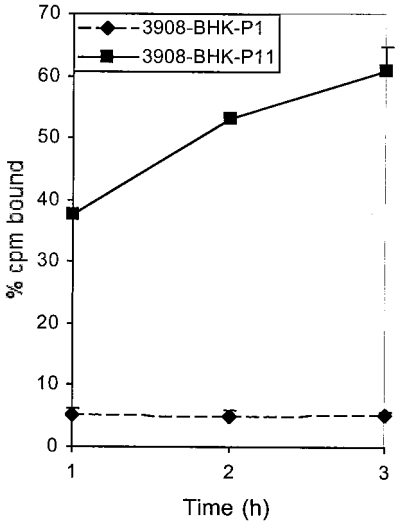

FIG. 8. Binding of purified, radiolabeled epizootic IC VEEV strain 3908 after 1 or 11 passages in BHK cells. Bars indicate standard deviations.

negatively charged than HS, and this difference may explain why all of the VEEV strains were inhibited almost equally by heparin.

Middle-American subtype IE VEEV strains. Enzootic and epizootic subtype IE VEEV strains were also evaluated for CHO cell binding. The enzootic subtype IE strain 68U201 showed higher binding levels than the enzootic ID strains, and the closely related epizootic IE strain CPA201 from the 1993 Mexican equine outbreak showed slightly greater binding (Fig. 7). Because the replacement of the negatively charged amino acid Glu by the positively charged Lys residue at position 117 of the E2 envelope glycoprotein was associated with the 1993 Mexican VEE epizootic and was shown to be responsible for the small-plaque phenotype (3), we evaluated GAG binding of strain CPA201, which was isolated from a horse and is known to be equine virulent (9). When compared to the closely related enzootic strain 68U201 from coastal Guatemala, CPA201 exhibited greater affinity for CHO cells (Fig. 7). When the E117K substitution was incorporated into the 68U201 backbone, the affinity of the mutant increased to nearly that of the epizootic strain CPA201, indicating that the E117K substitution increased GAG binding.

Effect of cell culture passage on GAG binding. Passage of alphaviruses, including VEEV, in cell culture is known to select for binding to HS, which can result in attenuation of the virus in mice. The mutations responsible for these changes include positively charged amino acids in the E2 envelope glycoprotein (2, 7, 8, 12, 13). Although there was no obvious correlation between cell culture passage history and CHO cell binding for the VEEV strains we evaluated, the passage history of some of the older (1960s and earlier) epizootic strains may be incomplete in our records. We therefore tested the effect of BHK cell passage on VEEV binding to CHO cells. When strain 3908 (wild-type virus derived from the first C6/36 cell passages) was passaged 10 times in BHK cells, its binding to CHO cells increased dramatically (from 5 to 6% to 37 to 61%) (Fig. 8), supporting the hypothesis that some of the older epizootic strains exhibited higher binding levels due to more extensive passage histories. This alteration in binding was accompanied by a Glu-to-Lys substitution at E2 amino acid position 3, a mutation within the furin cleavage site like those shown previously to mediate Sindbis virus binding to HS (12).

DISCUSSION

VEE emergence. VEE emergence depends on a combination of viral mutation and epidemiological and ecological events that must coincide in time and space. Mutations involving replacement of uncharged or negatively charged amino acids on the surface of the E2 envelope glycoprotein with positively charged Lys or Arg residues have been associated with significantly higher levels of equine viremia that result in efficient VEEV amplification (3, 25). These mutations typically produce a small-plaque phenotype that results from interactions between the positive charge on the VEEV virion and polyanions in unpurified agar (3, 14). E2 envelope glycoprotein mutations also result in more efficient infection of the epizootic mosquito vector, *Aedes taeniorhynchus*, suggesting that adaptation to mosquito vectors may also mediate VEE emergence (5).

Our results with natural enzootic and epizootic VEEV isolates indicate that the binding to GAGs, and more specifically to HS, is not consistently associated with the epizootic phenotype exhibited by VEEV. All experiments with low-passage strains, especially with viruses derived from cDNA clones, indicate that GAG binding plays little or no role in the epizootic phenotype. The strongest evidence against a role for GAG binding in VEE emergence comes from the epizootic subtype IC strain 3908, which was isolated during a major VEE epidemic/epizootic (26). After being passaged only once in C6/36 mosquito cells, this strain is highly virulent in experimental equine infections (R. A. Bowen, Colorado State University, unpublished data) and has several amino acid mutations lead-

ing to increased E2 charge (D117→G, E201→K, and T213→K) associated with VEE emergence from closely related enzootic ID strains (3) yet binds poorly to CHO cells. Although two of these three amino acid substitutions are similar in nature to those implicated previously in GAG binding, their positions are different and they clearly do not increase binding of strain 3908 compared to that of enzootic VEEV isolates. However, binding of strain 3908 to CHO cells did increase over that of enzootic strains after several passages in BHK cells, consistent with selection for artificial HS binding during cell culture passage. These results demonstrate that the positive-charge E2 mutations associated with the 1962 subtype IC VEE emergence are not the result of artifactual selection for GAG binding. Although many of the subtype IAB epizootic VEEV strains were isolated before the identification of enzootic variants in the 1960s (10, 23) and may therefore have a more extensive passage history, our results with more recent epizootic strains clearly indicate that the E2 mutations associated with epizootic emergence are not the result of extensive cell culture passage.

The role of GAG binding in VEE pathogenesis. The cellular and molecular effects of the positive-charge E2 mutations that accompany VEE emergence remain unknown but do not appear to involve consistently the binding to GAGs. One possible explanation for the epizootic/enzootic phenotypic difference is that the epizootic strains bind more efficiently to certain equine cells, such as dendritic cells, that could be responsible for viremia due to non-GAG interactions and/or that these mutations enhance VEEV binding to midgut epithelial cells of mosquito vectors. Another possibility is that these E2 mutations affect sensitivity to equine alpha/beta interferon (22). These hypotheses are being tested using infections with cDNA clones generated from enzootic VEEV strains and experimental equine and mosquito infections.

The strongest evidence that increases in GAG binding affect equine pathogenesis comes from the Mexican subtype IE strain CPA201, which was neurovirulent for one of three equines infected experimentally (9) and exhibits increased binding to CHO cells compared to the closely related enzootic strain 68U201, which is not associated with equine disease. The wild-type sequence at E2 position 117, which is responsible for the increased binding, is probably a mixture in virus populations isolated from equines (3). The role of this mutation in VEE emergence will therefore require detailed mutagenesis and equine virulence studies along with genetic analysis of natural VEEV populations from horses, mosquitoes, and reservoir hosts in Mexico.

ACKNOWLEDGMENTS

We thank Charles Calisher, Robert Shope, and Robert Tesh for providing many of the VEEV strains that we studied.

A.C.B. was supported by the James L. McLaughlin Infection and Immunity Fellowship Fund and the NIH Emerging Tropical Diseases T32 training grant AI-107526. A.M.P. was supported by a James W. McLaughlin Fellowship. This research was supported by National Institutes of Health grants AI39800 and AI48807 and by the National Aeronautics and Space Administration (NASA).

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