Heparan sulfate binding by natural eastern equine encephalitis viruses promotes neurovirulence

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The *Alphavirus* genus of the family Togaviridae contains mosquito-vectored viruses that primarily cause either arthritogenic disease or acute encephalitis. North American eastern equine encephalitis virus (NA-EEEV) is uniquely neurovirulent among encephalitic alphaviruses, causing mortality in a majority of symptomatic cases and neurological sequelae in many survivors. Unlike many alphaviruses, NA-EEEV infection of mice yields limited signs of febrile illness typically associated with lymphoid tissue replication. Rather, signs of brain infection, including seizures, are prominent. Use of heparan sulfate (HS) as an attachment receptor increases the neurovirulence of cell culture-adapted strains of Sindbis virus, an arthritogenic alphavirus. However, this receptor is not known to be used by naturally circulating alphaviruses. We demonstrate that wild-type NA-EEEV strain FL91-4679 uses HS as an attachment receptor and that the amino acid sequence of its E2 attachment protein is identical to those of natural isolates sequenced by RT-PCR amplification of field samples. This finding unequivocally confirms the use of HS receptors by naturally circulating NA-EEEV strains. Inactivation of the major HS binding domain in NA-EEEV E2 demonstrated that the HS binding increased brain replication and neurologic disease but reduced lymphoid tissue replication, febrile illness signs, and cytokine/chemokine induction in mice. We propose that HS binding by natural NA-EEEV strains alters tropism in vivo to antagonize/evade immune responses, and the extreme neurovirulence of wild-type NA-EEEV may be a consequence. Therefore, reinvestigation of HS binding by this and other arboviruses is warranted.

glycosaminoglycan | innate immunity | interferon

Eastern equine encephalitis virus (EEEV) is a positive-sense RNA virus in the genus *Alphavirus*, family *Togaviridae,* that is endemic in the Americas from southeastern Canada, the eastern seaboard and Gulf Coast of the United States, to the Caribbean and Central and South America. North American (NA)-EEEV strains cause periodic outbreaks of mosquito-borne encephalitis in humans and equines (1, 2), are highly neurovirulent, and, in comparison with related Venezuelan equine encephalitis virus (VEEV) and western equine encephalitis virus (WEEV), cause far more severe encephalitic disease in humans. Whereas VEEV and WEEV typically cause febrile illness that rarely progresses to encephalitis (3), NA-EEEV–infected adults experience sudden, but brief, prodromal symptoms such as fever, chills, myalgia, arthralgia, retro-ocular pain, headache, and decreased consciousness (1), and young humans often experience neurological symptoms without prodrome (3, 4). Neurological disease results in paralysis, seizures, coma, and death in 30–80% of patients, with mild to severe long-term neurologic deficits in an estimated 35% of survivors (3).

Although wild-type (WT) strains of both VEEV and NA-EEEV produce uniformly fatal disease in mice after subcutaneous (sc) inoculation (5–8), pathogenesis comparison reveals key differences that parallel distinguishing manifestations of severe human disease (8). VEEV-infected mice exhibit a biphasic disease course with a characteristic prodrome (rapid weight loss, hunched posture, decreased activity, and ruffling of the fur). In contrast, little or no prodromal phase is observed in NA-EEEV–infected animals, and seizures are frequently the first clinical sign of disease, progressing rapidly to severe encephalitis (8, 9). Likely underlying early febrile signs, VEEV targets dendritic cells and macrophages in the skin and subsequently in lymphoid tissues (10, 11), yielding high-titer serum viremia (5, 8, 12) and eliciting secretion of high levels of IFN-α/β and other cytokines into serum (5, 8, 13). Consistent with the lack of a prodrome in NA-EEEV–infected animals, lymphoid tissues are largely spared from virus infection or pathology (6, 8), and little or no systemic IFN-α/β is produced (8).

The neurovirulence of an arthritogenic alphavirus, Sindbis virus (SV), could be increased by introducing mutations into the E2 attachment glycoprotein that conferred efficient attachment to heparan sulfate (HS) (14). HS is a ubiquitous, sulfated polysaccharide glycosaminoglycan (GAG), which is expressed as a component of extracellular matrices and on cell surfaces (15, 16) and is used by some viruses as an initial cell attachment receptor (17). Multiple laboratory strains of arthropod-borne alphaviruses and flaviviruses have been shown to attach to this molecule (18–23). However, HS binding can be conferred by amino acid substitutions that arise in attachment proteins during in vitro passage (18, 24–27). It remains unclear whether any naturally circulating WT alphaviruses or flaviviruses attach to HS efficiently (13, 18). Herein, we demonstrate that natural NA-EEEV viruses use HS as an attachment receptor, which exacerbates neurovirulence while simultaneously limiting tropism for lymphoid tissue and prodromal disease in mice. These results indicate a role for HS binding in evasion/antagonism of immune responses and the severity of encephalitis caused by arboviruses.

Results

FL91-4679 Strain of NA-EEEV Binds to HS with Unique Efficiency Among WT Alphaviruses. To compare the dependence of in vitro infection of multiple New and Old World alphaviruses upon HS, we infected parental CHOK1 cells or pgsA745 GAG-deficient cells with GFP-expressing nonpropagative but replication-competent replicon particles (as described in ref. 8) derived from minimally in vitro-passaged strains of VEEV [Trinidad Donkey (TrD)], NA-EEEV (FL91-4679), WEEV (CB-87), SV (TR339), Ross River (RRV; T48), or chikungunya (CHIKV; La Réunion) viruses (Fig. 1*A*). No WT arthritogenic virus exhibited dependence upon HS for infection, exhibiting equal infectivity for both cell types ($P > 0.05$), whereas WT encephalitic New World viruses WEEV and VEEV exhibited intermediate dependence. NA-EEEV exhibited uniquely high dependence, being reduced in infectivity by ~90% on GAG-deficient cells—similar to the reduction observed for SV mutants with positively charged, cell culture-adaptive amino acid substitutions in the E2 glycoprotein (18, 28).

Comparison of E2 Glycoprotein Amino Acid Sequences Reveals Identity of Prototypic FL91-4679 Strain with Many NA-EEEV Strains Including Primary Unamplified Isolates. The HS binding phenotype is rapidly selected during in vitro propagation of arthropod-vectored RNA viruses (18, 23, 24), resulting in controversy regarding whether or not any WT RNA viruses bind HS. To address this

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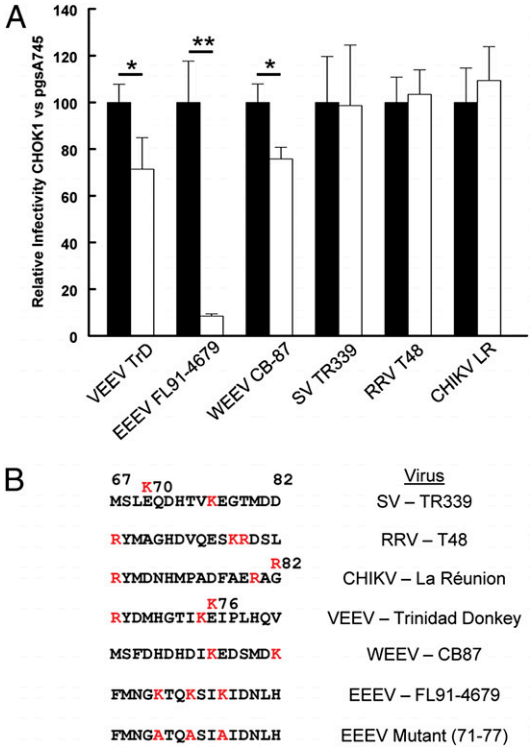

Fig. 1. HS dependence and E2 gene sequences of selected alphaviruses. (*A*) Relative infectivity of various normal (VEEV, CHIKV) or chimeric (SV, EEEV, WEEV, RRV) replicons for WT CHOK1 cells (filled boxes) or HS-deficient pgsA745 cells (open boxes). Infectivity of each replicon for CHOK1 cells was set to 100%. Error bars are SDs. *$P < 0.01$; **$P < 0.001$. (*B*) Comparison of the amino acid sequences of the indicated SV, RRV, CHIKV, VEEV, WEEV, WT EEEV, and mutant 71-77 EEEV strains between amino acids 67 and 82 of the mature E2 glycoprotein. Positively charged residues are shown in red. Substitution mutations to positively charged residues (24, 28, *) that confer HS binding to cell culture-adapted strains of SV, VEEV, or CHIKV are shown above the sequences.

issue with NA-EEEV, we aligned the translated E2 attachment protein sequence of the FL91-4679 strain used for the first cDNA clone of EEEV (29) with available E2 sequences of 61 other NA-EEEV strains, including 21 isolates from two New York clades that were sequenced directly from RT-PCR of tissues from infected crows, mosquitoes, and horses by Young et al. (30) and 40 strains subjected to minimal cultured cell amplification before sequencing (7, 30, 31). We found a remarkable conservation of E2 amino acid sequence over time (1933–2005) and between different geographical locations (Texas to Connecticut). There were only 35 total amino acid differences in the E2 glycoprotein between the 62 different isolates, 26 of which were found in the amino-terminal ectodomain of E2 implicated in cell attachment (amino acids 1–270; Table 1). Only 19 strains differed from FL91-4679 at any position in E2. Thus, FL91-4679 exhibited identity with the majority of all strains, including 20 of the 21 unamplified isolates, consistent with minimal variability in the nucleotide sequence of the NA-EEEV E2 gene noted previously (30, 31). Importantly, the sequence identity of FL91-4679 with unamplified primary isolates confirms high dependence upon HS for infectivity as the phenotype of naturally circulating NA-EEEV strains.

Ablation of Positive Charge in E2 71-77 Region Greatly Reduces HS Binding by NA-EEEV but Does Not Alter Replication Competence in Vitro. Multiple sites in E2 have been shown to be involved in HS binding by different alphavirus laboratory strains, including the positively charged furin protease cleavage sequence of PE2 (28), sites near the amino terminus (18, 24), in the region of amino acid 70 (18, 28), amino acid 114 (18, 24), and more carboxyl-terminal positions (25, 26, 32). However, mutation to positively charged residues in the region of E2 position 70 is common to multiple alphaviruses (24, 28). We compared this region between WT NA-EEEV, SV, VEEV, WEEV, RRV, and CHIKV and found a uniquely high concentration of uniformly spaced lysine residues in WT NA-EEEV (lysine at positions 71, 74, and 77; Fig. 1*B*). The three lysine residues were mutated to alanine in the FL91-4679 replicon glycoprotein helper and the FL93-939 full-length virus (which has E2 sequence identical to FL91-4679; ref. 7) to eliminate the positive charge of this region (mutant designated "71-77"). Infectivity assays using chimeric replicon particles coated in WT or 71-77 NA-EEEV glycoproteins and comparing CHOK1 cells with pgsA745 cells deficient in HS revealed that the mutagenesis had greatly reduced (~90%), if not eliminated, dependence of NA-EEEV infection upon HS (Fig. 2*A*)—similar to the difference between the non-HS-binding SV TR339 strain and the HS binding SV 39K70 mutant (18, 28). Blocking of virus HS binding sites by heparin competition and disruption of ionic interactions by infection in high-NaCl buffer indicated that infection by the WT NA-EEEV, but not the 71-77 mutant virus, was

Table 1. Amino acid changes in the E2 protein of NA-EEEV isolates compared with FL91-4679

Mutation from FL91-4679	E2 position	Strain*
Phe–Ser	6	TX95
Asp–Gly	58	TX95
Val–Ile	60	MA38
Asp–Glu	61	MA38
Lys–Thr	71	CT90, MS83
Lys–Gln	71	LA50, MA38
Thr–Lys	72	TX95
Asp–Glu	79	TX91
Pro–His	88	FL96
Ser–Phe	90	MA38
Leu–Val	91	FL96
Pro–His	117	FL96
Lys–Thr	137	FL96
Asp–Lys	146	MS83
Asn–Asp	150	NY71a
Thr–Ile	153	FL96
His–Tyr	161	NY74d
Leu–Ile	170	FL96
Ile–Met	186	MD90
Lys–Gly	199	MA38
Val–Ile	219	NY74b
Gln–His	221	FL02a
Lys–Arg	230	MD85
Pro–Leu	240	TX95
Val–Gly	253	NJ60
Pro–Leu	270	GA91
His–Tyr	276	Ten Broeck
Asp–Val	286	TX91
Thr–Ile	311	MA38
Leu–Met	321	NY69
Thr–Ile	324	MS83,TX91,TX95
Val–Ala	378	NY04k
Val–Ala	381	TX91
Cys–Phe	385	FL82
Ile–Leu	405	NJ60, NY69

Multiple viruses sequenced by direct amplification of natural isolates (NY 2003–2005 clades; ref. 30) have an identical sequence to FL91-4679 in the entire protein. Sequences are translated from nucleotide sequences in refs. 7, 30, and 31. National Center for Biotechnology Information nucleotide sequence accession numbers are given in these publications.
*State/year of isolation.

*Gardner CL, Klimstra WB, Ryman KD, Cell culture adaptation of chikungunya virus as a rational approach to live-attenuated vaccine design. 30th Annual American Society for Virology Meeting, July 16–20, 2011, Minneapolis, MN (abstr).

sensitive to both of these treatments (Fig. 2 *B* and *C*), which were previously shown to disrupt HS binding by SV strains (18, 19, 28). Finally, plaques formed on BHK-21 cell monolayers by the 71-77 mutant virus averaged 4.43 ± 0.19 mm in diameter compared with 6.41 ± 0.16 mm for the WT ($P < 0.001$)—similar to differences observed between HS-binding and nonbinding SV strains (14).

To assess the replication competence of the 71-77 mutant virus, we compared the specific infectivity with WT NA-EEEV and performed a single-step growth curve in BHK-21 cells (Fig. 2*D*). The specific infectivity of the non HS-binding 71-77 mutant virus was 16.1-fold lower than the WT ($P < 0.0001$)—similar to infectivity reductions when HS binding is eliminated from SV strains (14, 18, 28). In single-step growth curves, yields of infectious 71-77 mutant virus virions were reduced proportionally to the reduction in virus particle-specific infectivity for BHK-21 cells, suggesting that equal particle numbers are released. Together, these data suggest that lysine residues at E2 positions 71, 74, and 77 represent the major HS binding domain of WT NA-EEEV, and elimination of HS binding does not appear to compromise the replication competence of the virus beyond a reduction in per-particle infectivity for cultured cells.

Infection of Mice with NA-EEEV Glycoprotein-Targeted Virions Indicates That HS Binding Increases Neurovirulence and Brain Replication but Decreases Prodromal Signs and Lymphoid Tissue Replication. Eight-week-old CD-1 mice were infected either sc or intracranially (ic) with equal particle doses of WT and 71-77 viruses. Comparison of morbidity and mortality after sc infection with the WT and 71-77 viruses indicated that, although average survival times (AST) were similar (WT AST = 6.4 ± 1.5 d; 71-77 AST = 6.3 ± 0.7 d; $P > 0.05$), the 71-77 mutant virus caused significantly greater weight loss, which was observed initially on d 5 ($P < 0.05$) and continuing through d 6 and 7 postinfection (p.i.; $P < 0.01$), averaging a maximum of >30% for 71-77 vs. <10% for WT viruses (Fig. 3*A*). Furthermore, the 71-77 mutant caused obvious prodromal disease signs absent from the WT infection such as ruffling of fur, and disease was evident 48 h earlier in 71-77 mutant-infected mice.

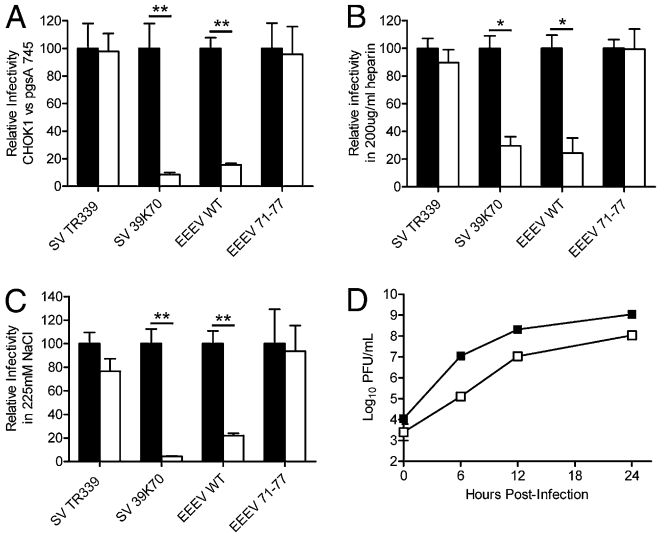

Fig. 2. HS dependence of WT and 71-77 NA-EEEV infectivity in comparison with WT SV TR339 and the HS binding SV 39K70 mutant. (*A*) Relative infectivity of replicons for WT CHOK1 (filled bars) and HS-deficient pgsA745 cells (open bars). (*B*) Relative infectivity of viruses for BHK cells in the presence of 200 μg/mL BSA (filled bars) or 200 μg/mL soluble heparin (open bars). (*C*) Relative infectivity of replicons for BHK-21 cells in RPMI infection medium supplemented to 220 mM NaCl (open bars). Infectivity for CHOK1 cells, infection in BSA, and normal RPMI (solid bars) was set to 100%. (*D*) Plaque titer data from a single-step (multiplicity of infection = 1) growth curve of WT and 71-77 mutant viruses on BHK-21 cells. WT, filled squares; 71-77, open squares. Error bars are SDs, and some are too small to be seen. *$P < 0.01$; **$P < 0.001$.

Notably, seizures were only observed with WT-infected mice (Fig. 3 *B* and *C*). After ic infection, the WT caused significantly more rapid mortality than the mutant (WT AST = 2.8 ± 0.3 d; 71-77 AST = 5.4 ± 0.5 d; $P < 0.001$), and similar differences in disease signs were observed (Fig. 3 *E* and *F*). We also constructed the 71-77 mutations in the background of a partially attenuated chimeric virus created by Weaver and colleagues (7), in which the nonstructural protein genes and *cis*-acting sequences of a mouse-avirulent South American EEEV strain were combined with the structural protein genes of WT NA-EEEV. The virus with WT E2 caused 100% mortality after ic inoculation (AST 5.5 ± 1.0 d), but introduction of the 71-77 mutation dramatically attenuated the neurovirulence of the chimera, resulting in 0% mortality.

Titration of virus from selected tissues revealed that the WT and 71-77 viruses replicated minimally in the draining lymph node (DLN) and disseminated to the local musculoskeletal tissue with similar kinetics. However, the mutant achieved higher and more sustained peak serum titers, reached the spleen earlier, and replicated to >1,000-fold higher titer in spleen by 48 h p.i. (Fig. 4 *A*–*D*). Although 71-77 virions were also detected in brain tissue earlier, replication plateaued between 24 and 48 h p.i., then increased, but again plateaued between 96 and 120 h p.i. In contrast, the WT virus exhibited an exponential increase in brain titer from initial detection through euthanasia of animals (Fig. 4*E*). After ic infection, the WT virus replicated to >10,000-fold higher titer (Fig. 4*F*) than the mutant. These results, combined with morbidity and mortality data, support a strong association of HS binding with neurovirulence.

Immunohistochemical staining for virus antigen in the brain on d 5 after sc infection paralleled the higher infectious virus titer of the WT but demonstrated that the viruses exhibited similar tropism (Fig. S1). H&E staining of contemporaneous spleen sections revealed that increased viral load in the 71-77 mutant-infected tissue was associated with greater depletion of cells in red pulp areas and increased signs of cell death (Fig. S2), resembling splenic changes observed after VEEV infection of mice (5), although milder.

To examine virus gene expression during in vivo infection without the possibility of spread of propagation-competent virus, we infected mice either sc or ic with equal particle numbers of chimeric VEEV–EEEV firefly luciferase (fLuc)-expressing replicon particles packaged in WT or 71-77 mutant E2 glycoproteins. fLuc expression in the brain (ic) or the footpad/popliteal lymph node (LN; sc) was examined by using in vivo imaging (IVIS) followed by quantitation of fLuc signal in tissues by in vitro luciferase assay. Chimeric replicon particles with a VEEV genome were used to distinguish the effects of HS binding from the previously observed inability of NA-EEEV to replicate in myeloid-lineage cells because of cell type-specific defective translation of the genomic RNA (8). Delivery by WT glycoproteins resulted in greater IVIS signal and fLuc gene expression in brain tissue (Fig. 5 *A*–*C*), in agreement with titration of propagation-competent virus after ic infection (Fig. 4*F*). Furthermore, although signal from both viruses was observed in the footpad after sc inoculation, fLuc expression from the 71-77 mutant virus was more readily observable in the popliteal LN (Fig. 5 *D* and *E*), confirmed by in vitro luciferase assay of removed LNs (Fig. 5*F*). Therefore, virus titration and chimeric replicon gene expression data are consistent in that HS binding enhances brain replication but suppresses virus dissemination to lymphoid tissues and/or subsequent amplification at these sites.

Cytokine and Chemokine Induction in Mice Is Altered by Elimination of HS Binding. Because mice infected with the 71-77 virus exhibited prodromal signs that, with VEEV, have been associated with lymphoid tissue replication and cytokine release (9, 13, 33), we examined cytokine and chemokine induction after sc infection with each virus. Consistent with previous findings (8), IFN-α/β was not detected by bioassay in the serum of WT-infected mice at any time p.i. However, this cytokine was detected in the serum of 71-77 mutant-infected mice at 24 and 48 h p.i. (Fig. 6*A*). Peak levels (~10^3 IU/mL) were substantially lower than those observed after mouse infection with VEEV (5, 8) but similar to those with SV (13). By ELISA, IFN-α (Fig. 6*B*) was detected in the serum of a single WT-infected mouse at 48 h p.i., whereas all 71-77–infected mice produced the cytokine at 48 h p.i. at significantly higher levels.

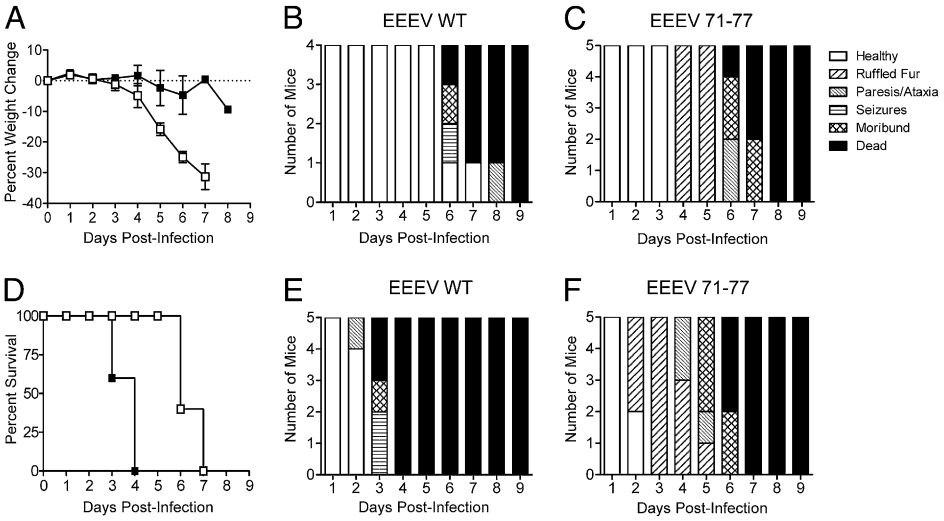

Fig. 3. Morbidity and mortality data for individual CD-1 mice inoculated with equal particles of WT NA-EEEV and the 71-77 mutant sc (*A–C*) or ic (*D–F*). WT, filled squares; 71-77, open squares (*A* and *D*). Seizures were defined as uncontrolled spasmodic limb and trunk movements, usually observed when mice had assumed a laterally recumbent posture. Seizures were measured for half-hour intervals during twice-daily collection of morbidity and mortality data. The presence of seizures with WT infection and absence with 71-77 mutant infection was reproduced in multiple independent experiments.

IFN-β levels were not significantly above mock-infected animals for the viruses at either time ($P > 0.05$). Differential lymphoid tissue targeting by the WT and mutant viruses was further implicated in the IFN-α/β induction differences because bioassay revealed significantly higher levels ($P = 0.04$) in the serum of mice inoculated sc with equal particles of the VEEV–EEEV chimeric replicons coated with 71-77 mutant or WT VEEV glycoproteins than when coated with NA-EEEV WT glycoproteins (Fig. 6*C*).

Induction of other cytokines/chemokines was initially compared in sera from WT NA-EEEV- and WT VEEV-infected mice 24 h p.i. by using the luminex bead assay (Fig. S3). As predicted (8), multiple proinflammatory cytokines/chemokines were induced to substantially greater levels after VEEV infection. Comparing the WT and 71-77 NA-EEEV viruses (Fig. S4), several cytokines associated with prodromal responses (e.g., IL-1-β, IL-17, TNF-α) were slightly, but significantly, higher in 71-77 mutant-infected mice, particularly between 24 and 72 h p.i., which coincided with the timing of maximal viral load in spleen. Notably, the level of T/NK cell chemotactic factor IP-10 (CXCL10; ref. 34) was greatly increased in serum of 71-77 mutant-infected mice at 48 h p.i. (Fig. S4), suggesting that HS binding may also suppress cell communication networks involved in adaptive immune clearance.

Discussion

The E2 glycoprotein amino acid sequences that we used for comparison with the FL91-4679 virus included directly sequenced RT-PCR products from unamplified mosquito, crow, and horse field samples collected in New York between 2002 and 2005 (30). Data of this type have not previously been available for determination of the E2 sequence of naturally circulating alphaviruses. This work confirms the use of HS as an attachment receptor by naturally circulating alphavirus strains, maps the HS binding domain of these viruses, and identifies a locus naturally variable in the NA-EEEV E2 protein that affects cell attachment and infectivity. Furthermore, we demonstrate that efficient HS binding contributes to both the high degree of neurovirulence of NA-EEEV and multiple unique aspects of disease including seizures, limited prodromal cytokine induction, and associated disease signs.

Another important aspect of these studies is the demonstration that HS binding suppresses WT NA-EEEV lymphoid tissue replication and exacerbates brain replication and neurovirulence. In mouse infections with chimeric VEEV–EEEV replicons using the myeloid cell-tropic VEEV genome, HS binding by WT NA-EEEV glycoproteins increased infectivity of viruses for brain tissue and simultaneously decreased infection of cells in the popliteal LN. Use of propagation-competent NA-EEEV viruses supported these results in that HS binding increased brain replication and greatly reduced detectable virus in spleen. Together, these results suggest that HS binding confers the ability to avoid infection of lymphoid tissues generally. Previous results from our laboratory indicated that WT NA-EEEV was defective in infection of naïve popliteal LN cells in vivo and several types of myeloid cells in vitro due to a de-

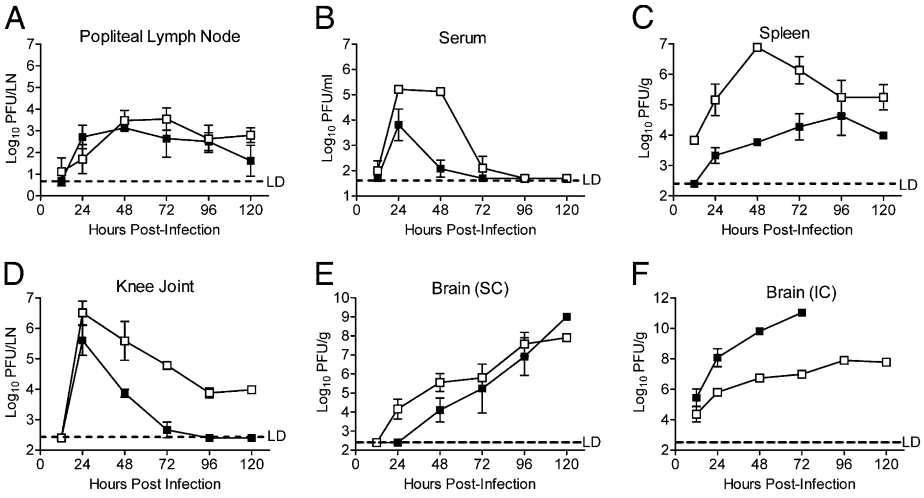

Fig. 4. Tissue plaque titer data from CD-1 mice infected sc (*A–E*) or ic (*F*) with equal particles of WT NA-EEEV or the 71-77 mutant. WT, filled squares; 71-77, open squares. LD, detection limit. Error bars are SDs, and some are too small to be seen.

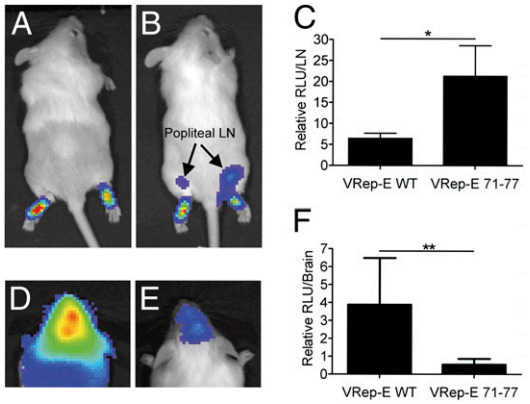

Fig. 5. Results of IVIS analysis of mice inoculated sc (*A* and *B*) or ic (*D* and *E*) with equal particles of fLuc-expressing chimeric replicons packaged in WT NA-EEEV (*A* and *D*) or 71-77 mutant (*B* and *E*) structural proteins. Quantitation of fLuc signal in homogenized brain (*C*) or DLN (*F*) is shown. Sensitivity of each image (footpad/DLN or brain) was set equally such that pseudocolor images of light emitted could be directly compared. Error bars are SDs, and some are too small to be seen. $*P = 0.01$; $**P = 0.01$.

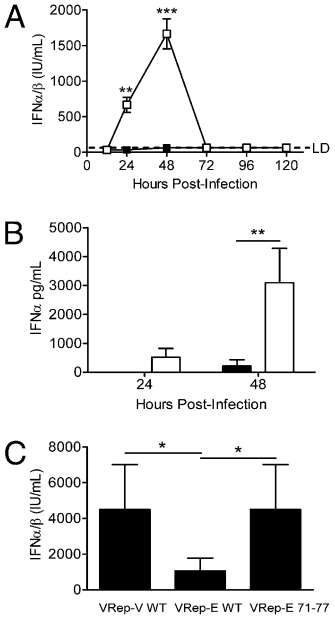

Fig. 6. Serum levels of IFN-α/β measured by bioassay (*A*; WT, filled square; 71-77, open square) or IFN-α ELISA (*B*; WT, filled bar; 71-77, open bar) at various times after sc inoculation of CD-1 mice with equal particles of WT EEEV or the 71-77 mutant or levels measured by bioassay 12 h after chimeric replicon infection of CD-1 mice (*C*). Error bars are SDs, and some are too small to be seen. LD, detection limit for the bioassay. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

ficiency in genomic translation initiation (8). The current studies are consistent with this finding in that neither WT nor HS binding-defective NA-EEEV viruses replicated efficiently in the DLN. In studies with SV and VEEV, HS binding increased the per-particle infectivity of virions for myeloid and other cells in vitro (8, 14, 18, 24), suggesting that deficiencies in lymphoid tissue replication for WT NA-EEEV in vivo are unlikely to be related to cell attachment and entry. The exact nature of tropism differences needs investigation, as does whether or not prodromal cytokines such as IFN-α/β observed in sera of HS binding-defective mutant-infected mice are released from infected myeloid cells. 71-77 virus infection elicited significantly higher levels of several cytokines/chemokines over the WT, including IFN-α/β, although levels were much lower than in VEEV-infected mice (this work and ref. 8). This result suggests that NA-EEEV phenotypes such as defective translation initiation in myeloid cells (8) also contribute to suppression of prodromal disease. However, the current results indicate that reduction in HS binding could be used to improve targeting of live-attenuated vaccine candidates derived from NA-EEEV or other HS binding viruses to some immune inductive sites concomitant with an increase in cytokine responses. They also raise the possibility of using HS analogs (e.g., ref. 35) as infection-blocking antiviral drugs during the acute phase of WT NA-EEEV infection.

Although HS binding alters replication efficiency and immune responses in vertebrates, the full role of HS binding in the sylvatic maintenance cycle of EEEV is not clear. Mouse infection studies using cell culture-adapted alphaviruses suggest that serum viremia is suppressed by efficient HS binding (18, 24, 32). Sustained viremia is likely important to the transmission of alphaviruses to arthropod vectors (3, 24); therefore, efficient HS binding would appear to conflict with this aspect of the arbovirus maintenance cycle. We propose that, with EEEV, the potential negative consequences of HS binding to viremic spread are compensated for by an increase in fitness in another aspect of the virus replication/transmission cycle. We have demonstrated that HS binding suppresses IFN-α/β responses and induction of other secreted host factors likely to be involved in virus clearance. Hypotheses that need to be explored further include: (*i*) HS binding is involved in suppression of the magnitude of acquired immune responses by limiting replication in lymphoid tissues; (*ii*) HS binding, either directly through increasing cell attachment or indirectly by modifying host cell responses to infection (as we demonstrated with SV; ref. 14), increases WT NA-EEEV replication within host tissues that may have a prominent role in generation and sustenance of viremia; or (*iii*) HS binding increases infectivity for or dissemination within arthropod vectors involved in EEEV enzootic or epizootic transmission and/or passerine birds, which serve as a primary reservoir for NA-EEEV.

Finally, as we have postulated previously (13), these studies further suggest that interaction with HS by native virus particles is a phenotype of WT encephalitic New World alphaviruses but not WT arthritogenic Old World alphaviruses. However, as we and others have demonstrated, HS binding can be conferred to the arthritogenic viruses by only a few passes in cultured cells (18, 24–26, 28). A similar analysis of directly PCR-sequenced field samples of arthritogenic alphaviruses should be completed to conclusively establish the phenotype of naturally circulating strains.

Methods

Sequence Analyses. Nucleotide sequences of the E2 gene from 62 strains of NA-EEEV (7, 30, 31) were downloaded from the National Center for Biotechnology Information and translated into amino acid sequences starting with the amino-terminal amino acid of the mature E2 protein. Amino acid sequences were aligned by using Vector NTI software (Invitrogen).

Viruses and Replicons. The VEEV ZPC738, and NA-EEEV FL93-939 and NA/SA chimeric virus cDNA clones (7) were gifts from Scott Weaver (University of Texas Medical Branch). The VEEV TrD-based replicon system was a gift from Robert Johnston (University of North Carolina). The construction of the chimeric replicon system derived from VEEV expressing eGFP or fLuc and packaged in VEEV TrD, SV TR339, and NA-EEEV FL91-4679 structural proteins has been described (8, 14). Other chimeric replicons were created similarly by PCR-amplification cloning of the capsid and/or PE2/6K/E1 genes of the SV 39K70 mutant (28), RRV T48 strain cDNA (RRV64; gift from Richard Kuhn, Purdue University), the WEEV CB87 strain structural protein gene cDNA (gift from Michael Parker, US Army Medical Research Institute of Infectious Diseases) into the chimeric helper cDNAs. CHIKV La Réunion strain-based replicons expressing eGFP were a gift from Stephen Higgs (University of Texas Medical Branch). 71-77 mutant viruses and replicon glycoprotein helpers were created by QuikChange II XL mutagenesis (Stratagene). Virus and replicon stocks were prepared and titered on BHK-21 cells as described (18, 36). BHK-21 cell plaque sizes were measured (20 plaques) on neutral red-stained monolayers. Infectious units in replicon stocks were determined via fluorescence microscopy (Olympus CKX41 microscope with Endow GFP filter set) for GFP or immunocytochemical staining for fLuc after BHK-21 cell infection as described (8). Virus-specific infectivities were determined as described (37), with the exception that a calculation of pfu/μg purified virus protein (BCS Protein Assay; Pierce) was used.

Virus Growth Curves and HS Interaction Studies. In single-step growth curves, BHK-21 cells were infected for 1 h at 37 °C at a multiplicity of infection of 1 pfu per cell, and supernatants were harvested for plaque assay at indicated intervals p.i. CHOK1 and GAG-deficient pgsA745 CHO cells were infected with equal dilutions of each GFP-expressing replicon, and infected cells were enumerated in triplicate wells via fluorescence microscopy at 18–20 h p.i. For heparin competition, viruses were reacted with indicated concentrations of heparin or BSA (Sigma) for 30 min on ice, and then the preparations were used to infect BHK-21 cells for 30 min on ice followed by washing with virus diluent (VD; OPTIMEM growth medium; Invitrogen) before overlay, transfer to 37 °C, and plaque assay. For infection in high ionic strength, viruses were diluted into RPMI, 1% donor calf serum (DCS) buffer (pH 7.0), supplemented to the indicated NaCl concentration, and cells were infected for 30 min at 37 °C followed by plaque assay.

Mouse Virus Infections and Pathogenesis Studies. Groups of five 8-wk-old CD-1 mice (Charles River Laboratories) were infected either ic or sc in both rear footpads with 10 μL of each diluted virus or 10 μL of VD by the same routes for mocks. Virus or replicon inocula containing equal particles were created by adjusting the dilution of titered virus/replicon stocks reflecting specific infectivity calculations for purified viruses such that equal virus protein was delivered. Virus- and mock-infected mice were observed at 24-h intervals until disease signs arose and 12 h thereafter. At each time, mice were scored for signs and weighed. ASTs and percent mortality (either mice that died or were euthanized) were calculated as described (38). In the experiment comparing VEEV and NA-EEEV WT, mice were infected sc with 1,000 BHK-21 pfu of each virus.

For plaque titration of tissues, mice were euthanized and immediately perfused with PBS containing 1% DCS. Blood was collected by cardiac puncture, and DLN, spleen, knee joint, and brain tissues were freeze-thawed, homogenized, and titrated by plaque assay. Tissues for sectioning were fixed (4% paraformaldehyde) before paraffin embedding, 5-μm sectioning, and H&E staining or immunocytochemistry. For virus staining, primary antibody was mouse anti-EEEV polyclonal ascites (ATCC), and secondary was goat anti-mouse-DyLight 594 (Jackson ImmunoResearch). Tissues were counterstained with DAPI (Invitrogen). All animal procedures were carried out in accordance with American Association for the Accreditation of Laboratory Animal Care International-approved institutional guidelines for animal care and use and approved by Institutional Animal Care and Use Committee.

In Vivo Bioluminescence Imaging and Data Quantification. Mice were infected ic or sc with equal particles of VEEV TrD replicons or VEEV-EEEV WT or VEEV-EEEV 71-77 chimeric replicons expressing fLuc as described above and imaged on a charge-coupled device (Xenogen) at 8 h p.i. as described (14). Relative intensities of transmitted luminescence were represented as a pseudocolor image ranging from violet (least intense) to red (most intense). Corresponding gray-scale photographs and color images were then superimposed. For fLuc quantitation, brains or DLNs were removed from mice followed by in vitro luciferase assay as described (16). Data for three mice per treatment are reported as mean values ± SD of the mean.

Mouse Serum Analysis. IFN-α/β in sera of infected mice was determined by biological assay as described (36). IFN-α and -β were measured separately by ELISA (PBL Biomedical). Other cytokines/chemokines were measured by using a mouse cytokine 20-plex polystyrene bead panel array (Invitrogen) read on a Bioplex 200 plate reader (Luminex) as per manufacturer's instructions.

Statistical Analyses. Statistical significance for mortality curves was determined by Mantel–Cox log-rank test, and for other experiments, a two-tailed Student *t* test with two-sample equal variance was used (GraphPad PRISM software).

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1. Deresiewicz RL, Thaler SJ, Hsu L, Zamani AA (1997) Clinical and neuroradiographic manifestations of eastern equine encephalitis. *N Engl J Med* 336:1867–1874.
2. Chénier S, et al. (2010) An eastern equine encephalomyelitis (EEE) outbreak in Quebec in the fall of 2008. *Can Vet J* 51:1011–1015.
3. Griffin DE (2007) *Fields Virology*, eds Knipe DM, Howley PM (Lippincott Williams & Wilkins, Philadelphia), pp 1023–1068.
4. Goldfield M, Sussman O (1968) Eastern encephalitis: a review of recent studies in New Jersey. *Res Publ Assoc Res Nerv Ment Dis* 44:164–177.
5. Charles PC, Trgovcich J, Davis NL, Johnston RE (2001) Immunopathogenesis and immune modulation of Venezuelan equine encephalitis virus-induced disease in the mouse. *Virology* 284:190–202.
6. Vogel P, Kell WM, Fritz DL, Parker MD, Schoepp RJ (2005) Early events in the pathogenesis of eastern equine encephalitis virus in mice. *Am J Pathol* 166:159–171.
7. Aguilar PV, et al. (2008) Structural and nonstructural protein genome regions of eastern equine encephalitis virus are determinants of interferon sensitivity and murine virulence. *J Virol* 82:4920–4930.
8. Gardner CL, et al. (2008) Eastern and Venezuelan equine encephalitis viruses differ in their ability to infect dendritic cells and macrophages: Impact of altered cell tropism on pathogenesis. *J Virol* 82:10634–10646.
9. Steele KE, Twenhafel NA (2010) Review paper: Pathology of animal models of alphavirus encephalitis. *Vet Pathol* 47:790–805.
10. MacDonald GH, Johnston RE (2000) Role of dendritic cell targeting in Venezuelan equine encephalitis virus pathogenesis. *J Virol* 74:914–922.
11. Nishimoto KP, et al. (2007) Restricted and selective tropism of a Venezuelan equine encephalitis virus-derived replicon vector for human dendritic cells. *Viral Immunol* 20:88–104.
12. Grieder FB, et al. (1995) Specific restrictions in the progression of Venezuelan equine encephalitis virus-induced disease resulting from single amino acid changes in the glycoproteins. *Virology* 206:994–1006.
13. Ryman KD, Klimstra WB (2008) Host responses to alphavirus infection. *Immunol Rev* 225:27–45.
14. Ryman KD, et al. (2007) Heparan sulfate binding can contribute to the neurovirulence of neuroadapted and nonneuroadapted Sindbis viruses. *J Virol* 81:3563–3573.
15. Esko JD, Lindahl U (2001) Molecular diversity of heparan sulfate. *J Clin Invest* 108:169–173.
16. Jackson RL, Busch SJ, Cardin AD (1991) Glycosaminoglycans: Molecular properties, protein interactions, and role in physiological processes. *Physiol Rev* 71:481–539.
17. Chen Y, Götte M, Liu J, Park PW (2008) Microbial subversion of heparan sulfate proteoglycans. *Mol Cells* 26:415–426.
18. Klimstra WB, Ryman KD, Johnston RE (1998) Adaptation of Sindbis virus to BHK cells selects for use of heparan sulfate as an attachment receptor. *J Virol* 72:7357–7366.
19. Byrnes AP, Griffin DE (1998) Binding of Sindbis virus to cell surface heparan sulfate. *J Virol* 72:7349–7356.
20. Germi R, et al. (2002) Heparan sulfate-mediated binding of infectious dengue virus type 2 and yellow fever virus. *Virology* 292:162–168.
21. Chen Y, et al. (1997) Dengue virus infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat Med* 3:866–871.
22. Liu H, Chiou SS, Chen WJ (2004) Differential binding efficiency between the envelope protein of Japanese encephalitis virus variants and heparan sulfate on the cell surface. *J Med Virol* 72:618–624.
23. Lee E, Lobigs M (2008) E protein domain III determinants of yellow fever virus 17D vaccine strain enhance binding to glycosaminoglycans, impede virus spread, and attenuate virulence. *J Virol* 82:6024–6033.
24. Bernard KA, Klimstra WB, Johnston RE (2000) Mutations in the E2 glycoprotein of Venezuelan equine encephalitis virus confer heparan sulfate interaction, low morbidity, and rapid clearance from blood of mice. *Virology* 276:93–103.
25. Heil ML, Albee A, Strauss JH, Kuhn RJ (2001) An amino acid substitution in the coding region of the E2 glycoprotein adapts Ross River virus to utilize heparan sulfate as an attachment moiety. *J Virol* 75:6303–6309.
26. Smit JM, et al. (2002) Adaptation of alphaviruses to heparan sulfate: Interaction of Sindbis and Semliki forest viruses with liposomes containing lipid-conjugated heparin. *J Virol* 76:10128–10137.
27. Lee E, Lobigs M (2002) Mechanism of virulence attenuation of glycosaminoglycan-binding variants of Japanese encephalitis virus and Murray Valley encephalitis virus. *J Virol* 76:4901–4911.
28. Klimstra WB, Heidner HW, Johnston RE (1999) The furin protease cleavage recognition sequence of Sindbis virus PE2 can mediate virion attachment to cell surface heparan sulfate. *J Virol* 73:6299–6306.
29. Platteborze PL, Kondig JP, Schoepp RJ, Wasieloski LP (2005) Comparative sequence analysis of the eastern equine encephalitis virus pathogenic strains FL91-4679 and GA97 to other North American strains. *DNA Seq* 16:308–320.
30. Young DS, et al. (2008) Molecular epidemiology of eastern equine encephalitis virus, New York. *Emerg Infect Dis* 14:454–460.
31. Brault AC, et al. (1999) Genetic and antigenic diversity among eastern equine encephalitis viruses from North, Central, and South America. *Am J Trop Med Hyg* 61:579–586.
32. Byrnes AP, Griffin DE (2000) Large-plaque mutants of Sindbis virus show reduced binding to heparan sulfate, heightened viremia, and slower clearance from the circulation. *J Virol* 74:644–651.
33. Tonkin DR, et al. (2010) Alphavirus replicon-based enhancement of mucosal and systemic immunity is linked to the innate response generated by primary immunization. *Vaccine* 28:3238–3246.
34. Dufour JH, et al. (2002) IFN-gamma-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. *J Immunol* 168:3195–3204.
35. Lee E, Pavy M, Young N, Freeman C, Lobigs M (2006) Antiviral effect of the heparan sulfate mimetic, PI-88, against dengue and encephalitic flaviviruses. *Antiviral Res* 69:31–38.
36. Yin J, Gardner CL, Burke CW, Ryman KD, Klimstra WB (2009) Similarities and differences in antagonism of neuron alpha/beta interferon responses by Venezuelan equine encephalitis and Sindbis alphaviruses. *J Virol* 83:10036–10047.
37. Klimstra WB, Nangle EM, Smith MS, Yurochko AD, Ryman KD (2003) DC-SIGN and L-SIGN can act as attachment receptors for alphaviruses and distinguish between mosquito cell- and mammalian cell-derived viruses. *J Virol* 77:12022–12032.
38. Klimstra WB, et al. (1999) Infection of neonatal mice with sindbis virus results in a systemic inflammatory response syndrome. *J Virol* 73:10387–10398.