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Replication and Clearance of Venezuelan Equine Encephalitis Virus from the Brains of Animals Vaccinated with Chimeric SIN/VEE Viruses

Slobodan Paessler,[1]* Haolin Ni,[1] Olga Petrakova,[2] Rafik Z. Fayzulin,[2] Nadezhda Yun,[1] Michael Anishchenko,[1] Scott C. Weaver,[1] and Ilya Frolov[2]

Center for Biodefense and Emerging Infectious Diseases, Department of Pathology, University of Texas Medical Branch, Galveston, Texas 77555-0609,[1] and Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas 77555-1019[2]

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Venezuelan equine encephalitis virus (VEEV) is an important, naturally emerging zoonotic pathogen. Recent outbreaks in Venezuela and Colombia in 1995, involving an estimated 100,000 human cases, indicate that VEEV still poses a serious public health threat. To develop a safe, efficient vaccine that protects against disease resulting from VEEV infection, we generated chimeric Sindbis (SIN) viruses expressing structural proteins of different strains of VEEV and analyzed their replication in vitro and in vivo, as well as the characteristics of the induced immune responses. None of the chimeric SIN/VEE viruses caused any detectable disease in adult mice after either intracerebral (i.c.) or subcutaneous (s.c.) inoculation, and all chimeras were more attenuated than the vaccine strain, VEEV TC83, in 6-day-old mice after i.c. infection. All vaccinated mice were protected against lethal encephalitis following i.c., s.c., or intranasal (i.n.) challenge with the virulent VEEV ZPC738 strain (ZPC738). In spite of the absence of clinical encephalitis in vaccinated mice challenged with ZPC738 via i.n. or i.c. route, we regularly detected high levels of infectious challenge virus in the central nervous system (CNS). However, infectious virus was undetectable in the brains of all immunized animals at 28 days after challenge. Hamsters vaccinated with chimeric SIN/VEE viruses were also protected against s.c. challenge with ZPC738. Taken together, our findings suggest that these chimeric SIN/VEE viruses are safe and efficacious in adult mice and hamsters and are potentially useful as VEEV vaccines. In addition, immunized animals provide a useful model for studying the mechanisms of the anti-VEEV neuroinflammatory response, leading to the reduction of viral titers in the CNS and survival of animals.

Venezuelan equine encephalitis virus (VEEV) is an enveloped virus with a nonsegmented, positive-sense RNA genome of approximately 11.4 kb and belongs to the *Alphavirus* genus in the *Togaviridae* family. The 5′ two-thirds of the genome contains four nonstructural proteins (nsP1 to nsP4) that form an enzyme complex required for viral replication (46–48). After release of the viral genome into the cytoplasm, a nonstructural polyprotein is translated directly from this RNA and utilized in the production of a full-length, negative-sense replicative RNA intermediate (45). The full-length RNA then serves as a template for the synthesis of positive-sense genomic RNA and for transcription of a subgenomic 26S RNA (46). The approximately 4-kb-long, subgenomic RNA corresponds to the 3′ one-third of the viral genome and is translated into a structural polyprotein that is proteolytically cleaved into the capsid and the envelope glycoproteins E2 and E1 (34). Two hundred forty copies of the capsid protein enclose the genomic viral RNA to form an icosahedral nucleocapsid that buds from the plasma membrane, acquiring a lipid envelope with embedded protein spikes formed by E1/E2 heterodimers (41, 48).

Venezuelan equine encephalitis virus is a zoonotic pathogen and a member of the VEE serocomplex, and it is divided into six distinct antigenic subtypes (54, 57, 58). Subtypes IAB and IC were previously associated with major epidemics and equine epizootics. In VEEV epizootics, equine mortality due to encephalitis can reach 83%. In 1995, a major outbreak occurred in Venezuela and Colombia, which was associated with the VEEV subtype IC. This epidemic resulted in roughly 100,000 human cases, with more than 300 fatal encephalitis cases estimated (35). Other recent epidemics indicate that VEEV still represents a serious public health problem (56). In humans, while the overall mortality rate is low (<1%), neurological disease, including disorientation, ataxia, mental depression, and convulsions, can be detected in up to 14% of infected individuals, especially children (21). Neurological sequelae in humans are also common (28). The predominant pathological findings in fatal human VEE cases include infections in (i) the central nervous system (CNS) (edema, congestion, hemorrhages, vasculitis, meningitis, and encephalitis), (ii) the lungs (interstitial pneumonia, alveolar hemorrhage, congestion, and edema), (iii) lymphoid tissue (follicular necrosis and lymphocyte depletion), and (iv) the liver (diffuse hepatocellular degeneration) (9, 10, 22).

A murine model for VEEV-induced encephalitis and lymphotropism is well established (7, 8, 18, 27). Subcutaneous infection of mice leads to biphasic disease with initial replication in lymphoid tissues, followed by viremia and penetration into and infection of the central nervous system (40), where the virus replicates until death of the infected animal occurs (12, 13, 16, 39). The infection of the CNS results in an acute meningoencephalitis that leads to the death of large numbers of neuronal cells and 100% lethality in mice (18, 27).

Syrian golden hamsters are also highly sensitive to VEEV infection but usually develop shock-like disease as a result of

* Corresponding author. Mailing address: Department of Pathology, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555-1019. Phone: (409) 747-0764. Fax: (409) 747-0762. E-mail: slpaessl@utmb.edu.

the direct destruction of the lymphoreticular system, ending in bacterial sepsis (14, 19, 20, 53). This makes the hamster an excellent second small animal model to study the relative pathogenicity of VEE-like viruses (strains or variants) as well as the safety and efficacy of potential vaccine candidates.

The live-attenuated VEEV TC83 vaccine strain (TC83) was developed in 1961 by serial passaging of the virulent VEEV subtype IAB Trinidad donkey (TRD) strain in guinea pig heart cell cultures; it remains the only strain available for human vaccination (4). More than 8,000 individuals have received TC83 in the past 4 decades (1, 6, 32). The cumulative data demonstrate that 20 to 40% of vaccinated people develop a disease with some symptoms typical of natural VEEV infection, including febrile, systemic illness and other adverse effects (1). Residual virulence has also been detected in mice, where TC83 is uniformly lethal for the C3H/HeN strain after intracerebral (i.c.) inoculation, and produces clinical illness in BALB/c and C3H/HeN mice for almost 14 days after subcutaneous (s.c.) inoculation (30). A formalin-inactivated version of the TC83 vaccine, C-84, is used for individuals who fail to seroconvert after vaccination with the standard formulation of TC83 (32). A more promising candidate vaccine, V3526, was described recently, in which attenuation of the TRD strain was achieved by introducing lethal mutations into the PE2 furin cleavage site of an infectious cDNA clone, followed by selection of a second-site suppressor mutation in the E1 glycoprotein gene (7, 17). This virus is highly attenuated in laboratory rodents. While this engineering minimizes the possibility of direct reversion to virulence, the potential for reversion to wild-type virulence via compensatory mutations remains unknown.

An alternative approach to the development of alphavirus vaccines is to create chimeric viruses that contain the replicative machinery from another alphavirus, Sindbis virus (SINV), and the structural genes from VEEV. The prototype chimeric virus SIN83 is capable of replicating in tissue culture and exhibits a safe and highly attenuated phenotype in mice and hamsters but induces a protective immune response against VEEV (31).

In this study, we describe the generation of novel chimeric SIN/VEE viruses with improved immunogenicity compared to that of SIN83. The safety and efficacy of these vaccine candidates were tested in murine and hamster models. Additionally, we describe a model to study the ability of the anti-VEEV response to reduce the viral titer in the murine brain as well as to evaluate different clinical and histopathological manifestations of the encephalitic response in vaccinated and unvaccinated mice. This model will be useful for determining the mechanisms responsible for protection against lethal encephalitis resulting from VEEV infection.

MATERIALS AND METHODS

Cell cultures. BHK-21 cells were kindly provided by Paul Olivo (Washington University, St. Louis, MO). Cells were maintained at 37°C in alpha minimum essential medium supplemented with 10% fetal bovine serum and vitamins.

Plasmid constructs. All of the plasmids were constructed by standard recombinant DNA techniques. The construct pSIN83 was described elsewhere (31). It contains, in 5′ to 3′ order, the promoter for SP6 RNA polymerase, followed by nucleotides (nt) 1 to 7601 of the SINV genome, nt 7536 to 11382 of VEEV TC83 (with an additional C→T mutation of nt 7555, aimed to preserve the secondary structure of the 5′ untranslated region [UTR] in the 26S RNA), followed by an AGGCCTTGGG sequence, a 355-nt sequence containing the SINV 3′ UTR (starting from nt 11394), and the poly(A) tail, followed by an XhoI restriction site. It also contained a single point mutation at nt 4065, changing Ser795 in nsP2 to Thr. SIN/TRD and SIN/ZPC constructs had essentially the same designs, but the protein-coding sequence of the subgenomic RNA was derived from the TRD and ZPC738 strains of VEEV, respectively. An additional SAAR/TRD chimera contained three mutations not present in SIN/TRD: A_5 to G mutation in the 5′ UTR, I_{538} to T in nsP1, and replacement of the termination codon between the nsP3 and the nsP4 genes by a cysteine codon. These mutations were previously described for the SAAR86 virus (44), a more pathogenic strain of SINV. Maps and sequences of the plasmids are available from the authors upon request.

RNA transcription and transfection. Plasmids were purified using standard protocols by centrifugation in cesium chloride gradients. They were linearized using the XhoI restriction enzyme site immediately downstream from the poly(A) sequences. RNAs were synthesized using SP6 RNA polymerase (Invitrogen) in the presence of a cap analog. The yield and integrity of transcripts were monitored by agarose gel electrophoresis in nondenaturing conditions. For electroporation, aliquots of transcription reactions were used without additional purification, and RNAs were transfected into BHK-21 cells by using previously described conditions (5). Viruses were harvested after development of cytopathic effects, usually at 24 h following electroporation.

Viral replication in cell culture. Cells were seeded at a concentration of 5×10^5 per 35-mm diameter dish. After 4 h of incubation at 37°C, monolayers were infected at a multiplicity of infection, as indicated in the figure legends, for 1 h at 37°C, and then the inoculum was replaced with 1 ml of complete medium. At selected times following infection, media were replaced, and titers of virus in the harvested samples were determined by a plaque assay on BHK-21 cells (3).

Animals. Pregnant, female NIH Swiss mice and 6-week-old female mice were purchased from Harlan (Indianapolis, Ind.). Newborn mice were maintained for 6 days after birth in an animal biosafety level 3 facility prior to experimental infection. Six- to 8-week-old female Syrian golden hamsters (*Mesocricetus auratus*) were purchased from Harlan and acclimatized in the facility for a week prior to infection.

Comparative virulence of TC83 and chimeric viruses in 6-day-old mice. (i) Survival study. Six-day-old mice (10 per group) were inoculated i.c. or s.c. with 20 μl of phosphate-buffered saline (PBS) containing 5×10^6 PFU of TC83 or chimeric SIN/VEE viruses. Animals were monitored twice daily for 2 months for paralysis, ataxia, and coma, clinical signs usually associated with VEEV-caused encephalitis.

(ii) Viral replication in the brain. Six-day-old mice (male and female) were inoculated i.c. with 20 μl of PBS containing 5×10^6 PFU of TC83 or chimeric SIN/VEE viruses. Brains were collected at selected dates from three animals per time point and sagittally sectioned in half. One half of each brain was homogenized in minimal essential medium containing 10% fetal bovine serum, and a 10% suspension was made. The organ suspension was maintained at −80°C until further processing. The titer of infectious virus was calculated using a plaque assay, as previously described (31).

(iii) RT-PCR for detection of viral nucleic acid. All reverse transcription (RT)-PCRs were carried out in 0.2-ml thin-walled PCR tubes using an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). All reactions were prepared in a final volume of 50 μl with identical reagents, as provided by Roche Applied Sciences (Indianapolis, Ind.), in the Titan One Tube RT-PCR kit (5 mM dithiothreitol, 200 μM deoxynucleoside triphosphates, 100 μM primers per tube, 5 units RNase inhibitor, 1 μl Titan enzyme mix, 5 μl of RNA template, 10 μl 5× RT-PCR buffer, and diethyl pyrocarbonate-treated water). The primers and testing RNA were mixed and denatured at 94°C for 2 min. Then, all other reagents were added into the tube. The RT-PCR program was 72°C for 2 min, 42°C for 20 min, 48°C for 20 min, 55°C for 20 min, and 94°C for 1 min, followed by 30 cycles as follows: 94°C for 20 s, 54°C for 20 s (temperature varied, depending on the primer used), and 68°C for 1 min (time was adjusted according to the DNA fragment size amplified). The primers used (VEE-1/cVEE-3, CW3W, and M2W) have been described previously (29).

(iv) Histopathology. The second half of the brain section was fixed in 4% buffered formalin for 48 h and stored in 70% ethanol for 12 h. The samples were then embedded in paraffin, sectioned (5 μm) and mounted on slides, and standard hematoxylin and eosin (H&E) staining was performed.

(v) Immunohistochemical analysis. Tissue sections (5 μm) were deparaffinized and rehydrated through xylene and graded ethanol solutions. Slides were then treated with 3% hydrogen peroxide with 0.05% sodium azide in PBS for 10 min, followed by microwave antigen retrieval at 100°C for 10 min in DAKO target retrieval solution (DAKO Corporation, Carpinteria, CA) in an H2800 microwave processor (Energy Beam Sciences, Inc., Agawam, MA). Following sequential 15-min incubations with 0.1% avidin and 0.01% biotin (Vector Lab-

oratories, Inc., Burlingame, CA) to block endogenous reactivity, slides were incubated in 0.05% casein (Sigma, St. Louis, MO)-0.05% Tween 20 (DAKO)-PBS for 30 min to block nonspecific protein binding.

(vi) T-cell staining. Brain tissue sections from infected or age-matched uninfected mice were immunostained for T cells using polyclonal rabbit anti-human CD3 antibody (DakoCytomation, catalog no. A0452). The cross-reactivity of this antibody with mouse CD3 has been demonstrated by the manufacturer and in previous studies (23, 24). Anti-CD3 antibody was applied to tissue sections at a 1:50 dilution for 60 min. Normal rabbit serum (DakoCytomation, catalog no. N1699) was used as a negative control antibody. Brain tissue from uninfected mice of identical age was used as the negative control tissue for immunostaining. Detection of anti-CD3 was performed utilizing biotin-conjugated goat anti-rabbit immunoglobulin (Ig) (absorbed against mouse antibody for detection of rabbit antibody on mouse tissue), followed by incubation with streptavidin-peroxidase according to the manufacturer's recommendations (HistoMouse-SP kit, catalog no. 95-9541; Zymed Laboratories Inc., South San Francisco, CA).

(vii) B-cell staining. Brain tissue sections from infected or age-matched uninfected mice were immunostained for B cells using purified rat anti-mouse CD45R/B220 monoclonal antibody (catalog no. 550286; BD Biosciences Pharmingen) (25) as follows. Primary antibody was applied to sections at 1:10 dilutions for 60 min. Subsequently, biotin-conjugated goat anti-rat IgG-specific polyclonal antibody (catalog no. 559286; BD Biosciences Pharmingen) was applied with the same concentration and incubation time. Normal rat serum (catalog no. X0912; DakoCytomation) was used as a negative control antibody in parallel immunostaining. The secondary, biotin-conjugated antibody was detected using the streptavidin-peroxidase reagent from the HistoMouse-SP kit (catalog no. 95-9541; Zymed) according to the manufacturer's recommendations.

(viii) Viral antigen staining. Mouse VEEV-specific hyperimmune serum raised against Everglades virus (a member of the VEE complex) (catalog no. VR1250AF; ATCC) was applied in a 1:200 dilution in PBS to brain sections for 60 min. Normal mouse serum (Ig ready-to-use kit, catalog no. N1698; DakoCytomation) was used as a negative control antibody in brains from infected mice. Brain sections from age-matched, uninfected mice were used as the negative control tissue for immunostaining. The HistoMouse-SP the kit (catalog no. 95-9541; Zymed) was used to detect mouse antibody on mouse tissue. Slides were counter-stained with Mayer's modified hematoxylin for microscopy.

Immunization using chimeric SIN/VEE viruses and challenge with virulent VEEV ZPC738 (ZPC738) in the murine model: viremia and the induction of serum-neutralizing antibodies. Six-week-old, female NIH Swiss mice (12 per group) were inoculated on day 0 s.c. into the medial thigh with chimeric SIN/VEE viruses at a dose of 5×10^5 PFU in a total volume of 100 μl of PBS. One half of the animals (six per group) received an additional booster on day 28, which was performed in the same way as the initial immunization. All of the animals were bled on days 1, 2, and 3 and at 4 and 8 weeks after immunization. Serum samples from the first 3 days after immunization were tested for the presence of infectious virus by a plaque assay on BHK-21 cells. Samples for serology were heat inactivated at 56°C for 30 min and stored at −70°C.

Antibody assays. Plaque reduction neutralization tests were performed on BHK-21 monolayers. A stock of TC83 virus was incubated for 1 h at 37°C with serial dilutions of serum from individual mice infected with chimeric viruses. Subsequently, cell monolayers were incubated with virus/serum mixtures for 1 h at 37°C, overlaid with 0.5% agarose, maintained for 36 h at 37°C, and stained with crystal violet (3). The serum dilution corresponding to an endpoint of 80% plaque reduction was determined.

Challenge studies to determine the protection against clinical encephalitis in the mouse model. Fifteen 6-week-old, female NIH Swiss mice were vaccinated with 5×10^5 PFU of each chimeric virus or PBS alone (control) in a total volume of 100 μl. After vaccination, each cohort of 15 animals was maintained for 8 weeks without any manipulation. Immunized animals were then challenged with VEEV subtype ID strain ZPC738 by using three different inoculation methods: (i) s.c. inoculation into the medial thigh with 10^6 PFU (roughly 10^6 50% lethal dose) per animal in 0.1 ml of PBS (five mice per group), (ii) i.c. inoculation into the left brain hemisphere with 2×10^5 PFU per animal in 20 μl of PBS (five mice per group), and (iii) intranasal (i.n.) inoculation with 2×10^5 PFU per animal in 20 μl of PBS (five mice per group). Mice were observed for clinical illness (for anorexia and/or paralysis) and/or death twice daily for a period of 2 months.

Challenge studies to determine protection against viral replication in the brain following i.c. or i.n. inoculation with ZPC738. A group of mice was vaccinated as described above, and the first challenge with ZPC738 was performed using two different inoculation methods: (i) i.c. inoculation into the left brain hemisphere with 2×10^5 PFU in 20 μl of PBS and (ii) i.n. inoculation with 2×10^5 PFU in 20 μl of PBS. Two animals per group were euthanized on days 3, 7, and 28 after infection, and lungs, livers, spleens, kidneys, and brains were collected for viral titration or histological examinations. In addition, 10 animals per group were housed for 28 days after i.n. challenge with ZPC738, without any manipulation. On day 28, all animals from this group received the second i.n. dose of 2×10^5 PFU of ZPC738. Two animals per group were euthanized on days 3, 7, and 28 postchallenge, and organs were collected as described above.

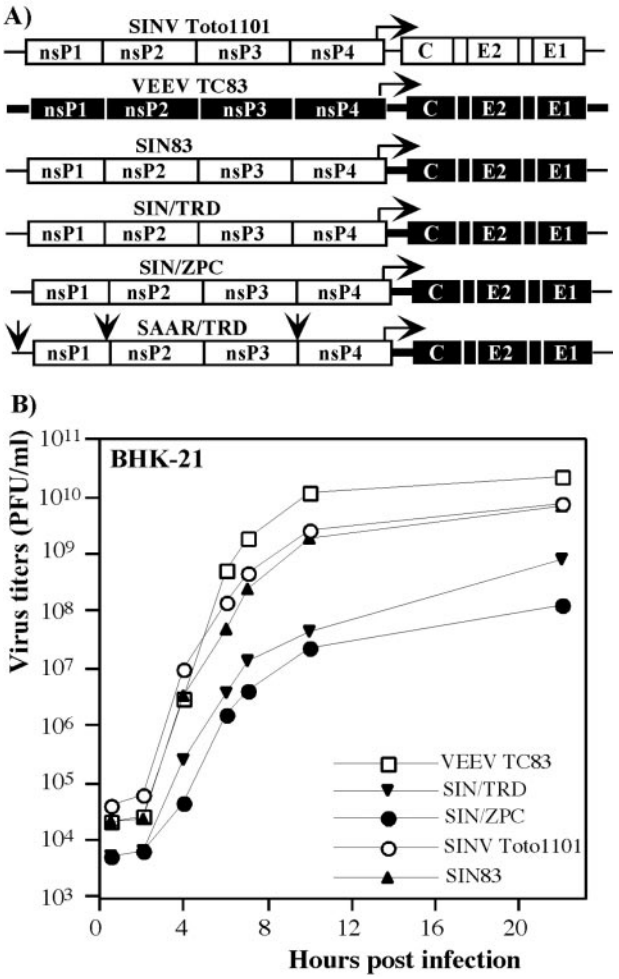

FIG. 1. Characteristics of viruses used in this study. (A) Schematic representation of parent and chimeric viruses. Bold, vertical arrows schematically present the positions of three mutations introduced into the SAAR/TRD chimera (in the 5′ UTR, in the nsP1, and between the nsP3 and the nsP4 genes). Horizontal arrows indicate the locations of the subgenomic promoter in the viral genomes. (B) In vitro replication of parent and chimeric viruses. Production of infectious virus in BHK-21 cells was measured at 0-, 4-, 8-, 12-, 16-, and 20-h time points after infection of monolayers with 10 PFU/cell (multiplicity of infection, 10).

Immunization and challenge of Syrian golden hamsters. Three 6-week-old female Syrian golden hamsters per viral strain were vaccinated s.c. in the right medial thigh with 5×10^5 PFU of SAAR/TRD, SIN/ZPC, SIN/TRD, or TC83 strain or PBS alone. Blood samples were obtained daily for the first 3 days after infection, and the animals were observed twice daily for 21 days. Serum viremia was determined by using a plaque assay on BHK-21 cells as previously described. The presence of neutralizing antibody in hamster serum samples was determined via a plaque reduction neutralization test, as described for the murine experiments. Three weeks after vaccination, the hamsters were challenged s.c. in the medial thigh with ZPC738 at a dose of 10^6 PFU in a total volume of 100 μl of PBS (roughly 5×10^6 50% lethal dose). The animals were observed for 28 days, and deaths or cases of clinical illness were documented.

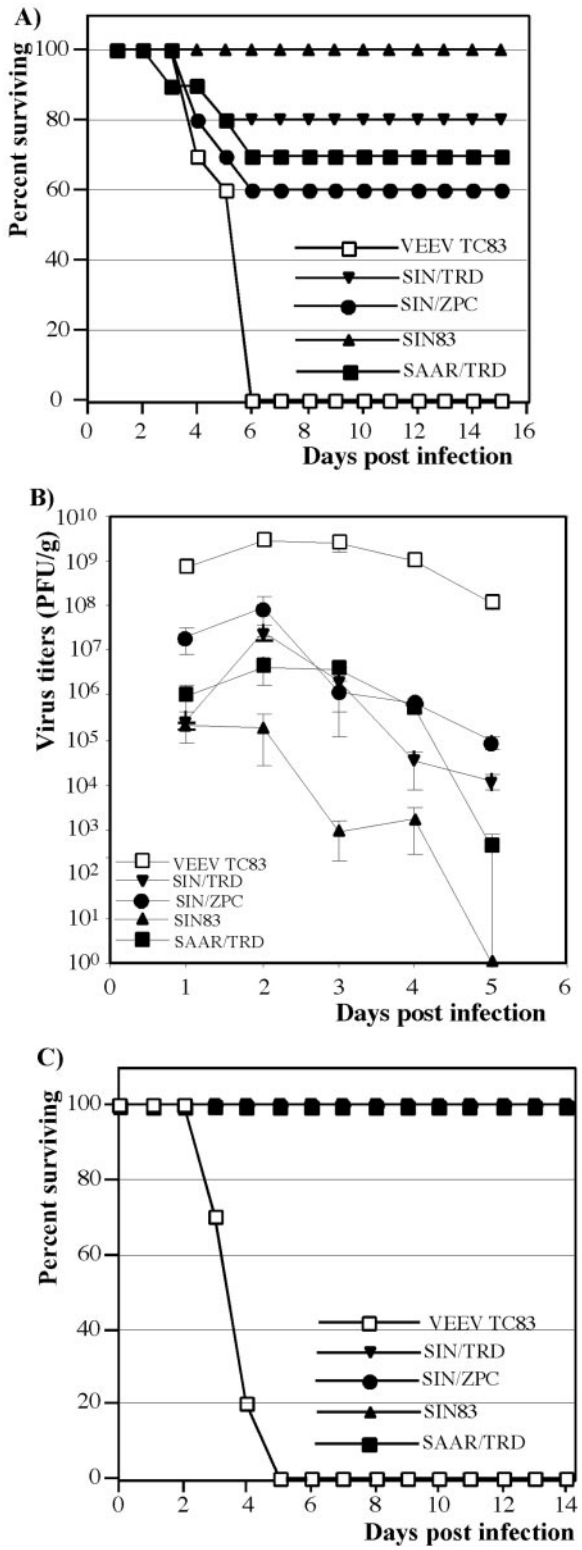

FIG. 2. Comparison of CNS infection with different viruses in 6-day-old NIH Swiss mice. Six-day-old mice (10 per group) were inoculated i.c. or s.c. with 20 μl of PBS containing 5×10^6 PFU of TC83 or chimeric SIN/VEE viruses. (A) Animal mortality following i.c. inoculation with chimeric SIN/VEE viruses or TC83. To evaluate the virulence of chimeric SIN/VEE viruses in comparison to the vaccine strain, TC83, mice were inoculated with SIN/TRD, SIN/ZPC, SIN83, SAAR/TRD, or TC83 and monitored twice daily for 2 months, and deaths were recorded. (B) Viral replication in the brain. Mice infected with parent or chimeric viruses via i.c. route (shown in panel A) were sacrificed (three per group) on days 1 to 5 postinfection. Brains were harvested, and homogenized tissues were used in a plaque assay performed in BHK-21 cells to quantify viral titers (PFU per gram of brain tissue). (C) Animal mortality following s.c. inoculation with TC83 or chimeric SIN/VEE viruses. To evaluate the safety of chimeric SIN/VEE viruses in comparison to that of TC83, mice were inoculated via s.c. route with 5×10^6 PFU of SIN/TRD, SIN/ZPC, SIN83, SAAR/TRD, or TC83 and monitored twice daily for 2 months, and deaths were recorded.

RESULTS

Recombinant viruses. In our previous study, we designed a chimeric SIN83 virus containing all of the structural proteins of TC83. This virus was highly attenuated in the murine model but was capable of inducing an efficient immune response to protect against subsequent infection with ZPC738. The main drawback to using this prototypic chimera was that the TC83-specific structural genes contained a number of mutations adapting this virus to replication in tissue culture but reducing its replication in vivo (31). These same mutations were also found to affect the antigenic structure of VEEV, making it different from the parental strain, VEEV TRD (36, 37). Therefore, we designed a series of SIN/VEE chimeras carrying structural proteins of various VEEV strains. The design of the chimeric viruses used here was essentially the same as that previously described for SIN83 (31). The SIN/ZPC-encoded glycoproteins were derived from the enzootic strain VEEV ZPC738 (ID subtype), and SIN/TRD-expressed glycoproteins were derived from the epizootic strain VEEV TRD (the parental strain used for TC83 development, an IAB/C subtype). The SAAR/TRD chimera was designed to determine whether the most virulent SINV backbone might lead to an increase in pathogenesis or, alternatively, may exhibit improved replication and immunogenicity. Three SAAR86-specific mutations were introduced into the SINV Toto1101 backbone (Fig. 1).

The in vitro-synthesized RNAs for all constructs were transfected into BHK-21 cells to generate the primary stocks of the viruses. In parallel experiments, we assessed the infectivities of the RNAs by a standard plaque assay on BHK-21 cells. RNAs generated from all of the constructs were as infectious as those synthesized for TC83, SIN83, and Toto1101 (Fig. 1B), indicating that no mutations in the constructs were required for replication of the new chimeras in tissue culture. As with the parental VEEV strains, SIN/TRD and SAAR/TRD in particular demonstrated a small plaque phenotype and replicated to lower titers than did the parent SINV in tissue culture. However, the observed low titers may be a reflection of limited infectivity for the cells rather than less-efficient replication, as has been reported previously for other non-culture-adapted alphaviruses (26). The high-titer stocks of all of the viruses were used for the animal studies.

Chimeric viruses are highly attenuated in 6-day-old mice. One of the main objectives of our study was to assess the potential virulence of the designed chimeric viruses. Their structural proteins were derived from the most pathogenic strains of VEEV that cause encephalitis in mice of any age after inoculation via any route. To evaluate the virulence of all

of the newly designed recombinant variants in comparison to the TC83 vaccine strain and the previously described SIN83 chimera, we inoculated 6-day-old NIH Swiss mice with high doses (5×10^6 PFU) of infectious viruses (Fig. 2A and B). As previously reported, TC83 caused death within 4 to 6 days postinfection while the SIN83 virus was completely attenuated, with 100% survival of the infected mice. All other recombinant viruses produced intermediate mortality rates (Fig. 2A). All brains from animals that died contained infectious virus, whereas no virus was recovered from brains of animals that were sacrificed 2 months after inoculation (data not shown). The same chimeric viruses were completely attenuated for 6-day-old mice after s.c. inoculation (Fig. 2C) or for 6-week-old mice via any inoculation route (data not shown). Animals inoculated via s.c. route did not develop any detectable neurological signs and survived for 60 days after vaccination. Taken together, these results indicated that the SIN/VEE chimeric viruses were attenuated in the murine model even though they contained the structural genes from virulent enzootic and epizootic strains of VEEV. The mutations in the SINV nonstructural genes that were introduced into the SAAR/TRD chimera, which could potentially lead to a higher virulence of the chimeric virus, were also insufficient to modify pathogenicity in mice.

Replication levels of the recombinant viruses in the brains of 6-day-old NIH Swiss mice were further investigated by quantifying infectious virus recovered from the brains of animals infected via i.c inoculation: TC83 replicated to the highest titers, and the animals were incapable of clearing the infectious virus before death; the SIN83 virus replicated to the lowest titers, and within 5 days postinfection, infectious virus in the brain was below the limit of detection; and the infectious SIN/ZPC, SAAR/TRD, and SIN/TRD chimeric viruses, which replicated at intermediate levels, had highly reduced titers on day 5 (Fig. 2B), and the infectious virus was undetectable in the brains of all of the survivors (2 months after infection). The viral titers in the brain were inversely correlated with the days of survival.

To confirm encephalitis as a cause of death and to monitor the histological characteristics of inflammation in the brains of animals that survived i.c. infection with the chimeras, we performed detailed pathological examinations. Inflammatory responses were detected in all animals that died, regardless of the infecting virus. The inflammation was characterized by perivascular and vascular infiltration of mononuclear cells, edema, microhemorrhages, and neuronal cell death. Figure 3 demonstrates pathological changes in the brains of TC83-infected animals. The neuroinflammatory response in these mice was disseminated, and on days 4 and 5 postinoculation (p.i.), all parts of the brain were equally affected. Mice infected with the chimeras developed a more focal encephalitic response, mainly in the cortex (data not shown).

In addition to performing histopathological studies, we analyzed the distribution of viral antigens in the brains. In all animals inoculated i.c. with chimeras or TC83, we detected antigen in the cortex within the first 2 days p.i. However, at 5 to 6 days p.i., VEEV-positive cells were found in brain sections of only the TC83-infected mice. Viral antigen was always detected in the cytoplasm of neurons and in neuropil, indicating productive infection of this cell type (Fig. 4) and potential

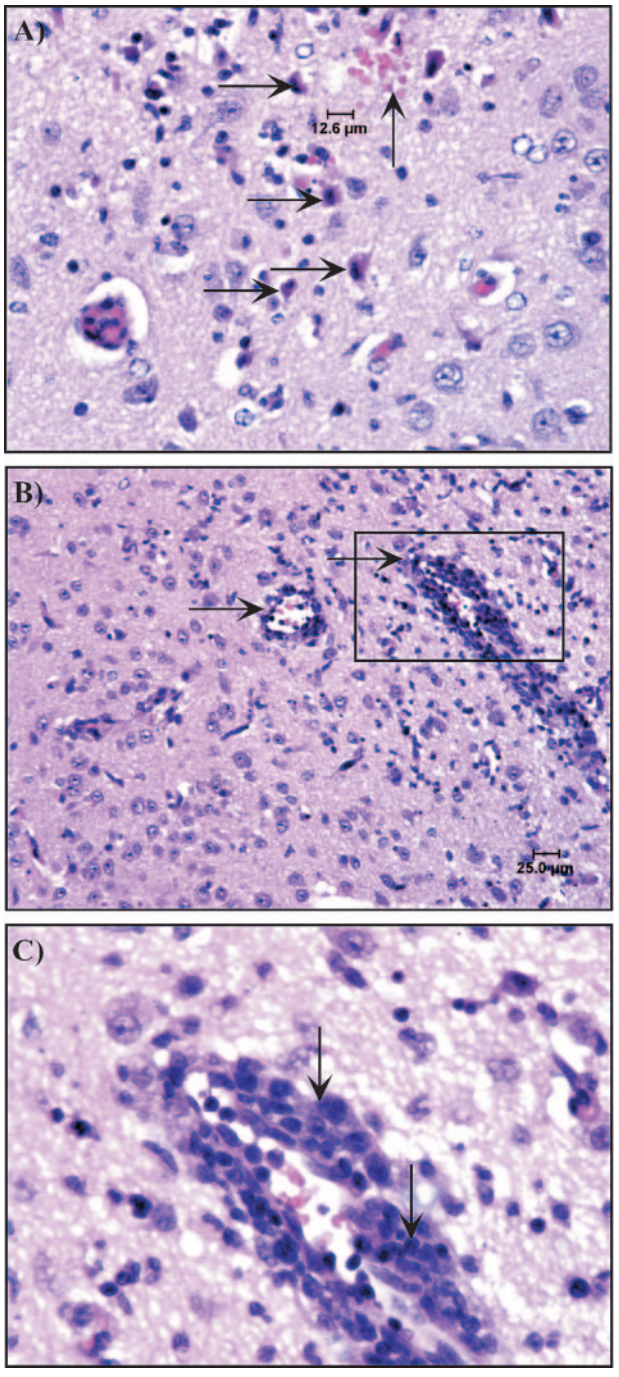

FIG. 3. Histopathological analysis of brains from mice infected with TC83. Representative photomicrographs of H&E-stained brain of a 6-day-old NIH Swiss mouse inoculated with TC83 and tissue obtained at 5 days following infection with TC83 virus are presented. (A) Neuronal cell death characterized through angulation of hypereosinophilic neurons (horizontal arrows) and microhemorrhage (vertical arrow) in the cortex. (B) Vascular and perivascular infiltration by mononuclear cells (arrows). (C) Higher magnification image of that shown in panel B.

axonal dissemination within the brain regardless of the virus strain. These results indicate that all recombinant viruses were highly attenuated in the 6-day-old mice and were completely attenuated in adult animals. All viruses were capable of initi-

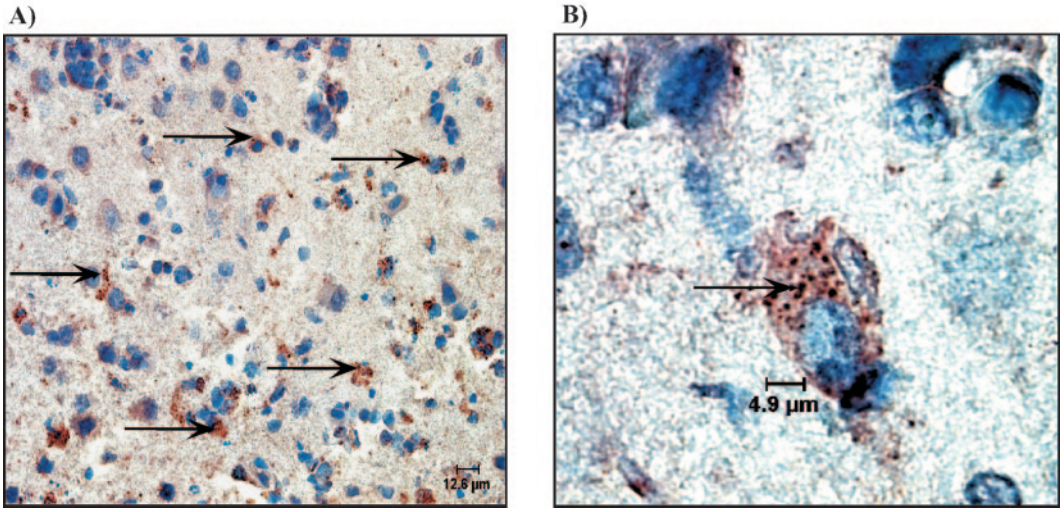

FIG. 4. Immunohistochemical analysis of the brain from a SAAR/TRD-infected mouse. Representative photomicrographs were obtained from an immunohistological analysis of brains obtained at 4 days following infection of 6-day-old NIH Swiss mice with SAAR/TRD. The localization of viral antigen was demonstrated via immunostaining of brain sections using antibody to VEEV (catalog no. VR1250AF; ATCC). VEEV antibody was detected using biotinylated secondary antibody, followed by avidin-peroxidase color development. Slides were counter-stained with Mayer's modified hematoxylin prior to mounting and microscopy. (A and B) Viral antigen in brains of SAAR/TRD-inoculated mice. Viral antigen was detected in association with neuronal cells and neuropil (horizontal arrows) in the midbrain. (B) Higher magnification image of that shown in panel A. The cytoplasmic distribution of the viral antigen in this neuronal cell is indicated by the horizontal arrow.

ating replication in the brains of 6-day-old mice after i.c. inoculation. Further, we observed a rapid reduction in levels of infectious virus and an increase in survival time for animals inoculated with chimeric viruses.

Viremia and induction of serum-neutralizing antibodies using chimeric SIN/VEE viruses. To assess the immunogenicity of the chimeras described in this study, we inoculated 6-week-old female mice s.c. (5×10^5 PFU), either on day 0 only or on days 0 and 28. The viremia levels were determined on days 1, 2, and 3 p.i., and the production of serum antibodies with VEEV-neutralizing activity (Ab_{neut}) was measured at 4 and 8 weeks p.i. Four recombinant viruses were tested: SIN83, SIN/TRD, SAAR/TRD, and SIN/ZPC. No infectious virus was detected on days 1 through 3 in the serum samples of animals inoculated with chimeric viruses ($n = 12$). However, all of the viruses induced serum Ab_{neut} (Table 1). A booster on day 28 did not significantly increase titers of Ab_{neut} in the serum, except in that of animals that received the SIN/ZPC virus. The SIN/ZPC virus induced noticeably higher titers of Ab_{neut}, and the boost immunization additionally increased Ab_{neut} levels. This difference might be explained by the ability of ZPC738 to replicate efficiently in wild rodents, which is a distinguishing characteristic of enzootic viruses (55), and the fact that glycoprotein-coding genes were derived from the genome of this virus after a minimal number of passages in the cell culture (2).

TABLE 1. Titers of neutralizing antibody in vaccinated mice

Virus strain	Titer of antibody (mean ± SD) after[a]:			
	Single immunization[b]		Booster immunization[c]	
	4 wk p.i.	8 wk p.i.	4 wk p.i.	8 wk p.i.
SIN83	55 ± 73	73 ± 76	100 ± 40	160 ± 120
SIN/TRD	37 ± 23	57 ± 27	50 ± 55	73 ± 47
SAAR/TRD	126 ± 53	167 ± 89	160 ± 113	152 ± 107
SIN/ZPC	187 ± 109	253 ± 205	253 ± 106	487 ± 254

[a] The reciprocal titer of the serum capable of neutralizing 80% of PFU of TC83 virus in a plaque reduction neutralization test.

[b] Six-week-old, female NIH Swiss mice (12 per virus strain) were inoculated on day 0 s.c. into the medial thigh with the indicated chimeric SIN/VEE virus at a dose of 5×10^5 PFU in a total volume of 100 μl of PBS.

[c] Six-week-old, female NIH Swiss mice (six per virus strain) received an additional booster on day 28 s.c. into the medial thigh with the indicated chimeric SIN/VEE virus at a dose of 5×10^5 PFU in a total volume of 100 μl of PBS.

Challenge studies to determine the protection against clinical encephalitis in the mouse model. After validating attenuation and demonstrating immunogenicity in the mouse model, we tested the capability of the chimeras to induce protection against clinical encephalitis caused by ZPC738, a virulent strain of VEEV. Mice were immunized with a single s.c. dose of 5×10^5 PFU of chimeric SIN/VEE viruses or TC83 virus. The mock-vaccinated mice were inoculated via s.c. route with PBS only, as indicated in Materials and Methods. At 8 weeks following vaccination, all animals (five per experimental group) were challenged with ZPC738 using one of three different inoculation routes (s.c., i.c., or i.n.) and were monitored twice daily for 28 days. Irrespective of the route of challenge, all mock-vaccinated animals rapidly developed clinical symptoms of encephalitis and died. Animals vaccinated with SIN/TRD, SIN/ZPC, SAAR/TRD, or TC83 remained free of clinical signs of encephalitis throughout the study (Table 2) and were euthanized 28 days after challenge. In contrast, some of the animals vaccinated with SIN83 and challenged via i.c. or i.n. route with ZPC738 developed clinical encephalitis (two out of five animals) and died (one out of two animals) (Table 2). Peripheral challenge (s.c.) did not produce clinical signs of encephalitis or mortality in any of the animals. These data suggested that the immune response caused by the SIN83 virus was less efficient than that induced after immunization with other chimeras. In spite of its superior attenuation, SIN83 only

TABLE 2. Challenge studies to determine protection against clinical encephalitis and death in mice vaccinated with chimeric SIN/VEE viruses

Vaccine group[a]	Clinical outcome after inoculation challenge (no. of mice)[b]					
	Encephalitis			Death		
	s.c.	i.c.	i.n.	s.c.	i.c.	i.n.
SIN83	0	2	1	0	1	0
SIN/TRD	0	0	0	0	0	0
SIN/ZPC	0	0	0	0	0	0
SAAR/TRD	0	0	0	0	0	0
Mock[c]	5	5	5	5	5	5

[a] Six-week-old, female NIH Swiss mice (15 per virus strain) were inoculated on day 0 s.c. into the medial thigh with the indicated chimeric SIN/VEE virus at a dose of 5×10^5 PFU in a total volume of 100 μl of PBS or PBS alone.

[b] Encephalitis (paralysis and/or ataxia) or death following s.c. challenge (five per group) with ZPC738 at a dose of 10^6 PFU per animal in 100 μl and i.c. (five per group) or i.n. (five per group) challenge with ZPC738 at a dose of 2×10^5 PFU per animal in 20 μl of PBS at 8 weeks following vaccination with the indicated chimeric SIN/VEE virus.

[c] PBS alone.

partially protected mice against i.n. and i.c. challenges with high doses of ZPC738. Thus, SIN83 is not likely to be the most efficient vaccine candidate.

ZPC738 replicates in the brains of immunized mice without causing clinical encephalitis. After confirming the high level of protection against encephalitic disease in the three challenge models, we compared the abilities of SIN/ZPC, SIN/TRD, SAAR/TRD, SIN83, and TC83 to protect against ZPC738 replication in the brain. Following i.c. or i.n. challenge, ZPC738 replicated in the brains of all 6-week-old mice previously immunized with chimeric SIN/VEE viruses or TC83 virus, despite the lack of clinical signs of encephalitis. All animals challenged via i.n. route had similarly high titers of infectious virus in their brains on day 3 postchallenge, regardless of whether they were immunized prior to infection. By contrast, mice that had been immunized with SIN/ZPC, SIN/TRD, SAAR/TRD, or TC83 had undetectable or highly reduced viral titers in the brain on day 7 postchallenge via i.n. route (Fig. 5B). However, encephalitis-free mice that had been immunized with SIN83 and euthanized on day 7 postchallenge had titers of virus in the brain similar to those of mock-immunized animals that were terminally ill at that time point or had higher titers of virus than the mock-immunized mice (Fig. 5B). We also detected infectious ZPC738 in the lungs, livers, and kidneys of some animals on day 3 postchallenge (data not shown).

Further, we performed histopathological studies to determine whether a correlation existed between viral replication, pathology, and inflammation in the brain tissues of mice following vaccination and subsequent challenge with ZPC738. Histopathological findings suggested critical differences in the inflammatory response in the brain, despite the lack of difference in titers of ZPC738 (Fig. 5) or viral antigen distribution between vaccinated and mock-vaccinated animals (Fig. 7). On day 3 postchallenge, a dense inflammatory cell infiltrate was observed in the brains of vaccinated mice (Fig. 6A to C), among which B and T cells could be detected via immunohistochemical staining (Fig. 7). In contrast, these infiltrates were absent in the mock-vaccinated mice, and no B or T cells could be detected on day 3 postchallenge (Fig. 7). The infiltrates were correlated with disseminated encephalitis and meningitis (Fig. 6A to C). On day 7 postchallenge, we detected histopathological encephalitis characterized by vascular and perivascular mononuclear cell infiltration and activation of microglia in the brains of all animals. In addition, regardless of the immunization history, all animals had detectable B and T cells in the brain. At day 7 postchallenge, we were unable to detect any viral antigen in the brains of vaccinated mice (Fig. 7), whereas disseminated viral antigen was detected throughout

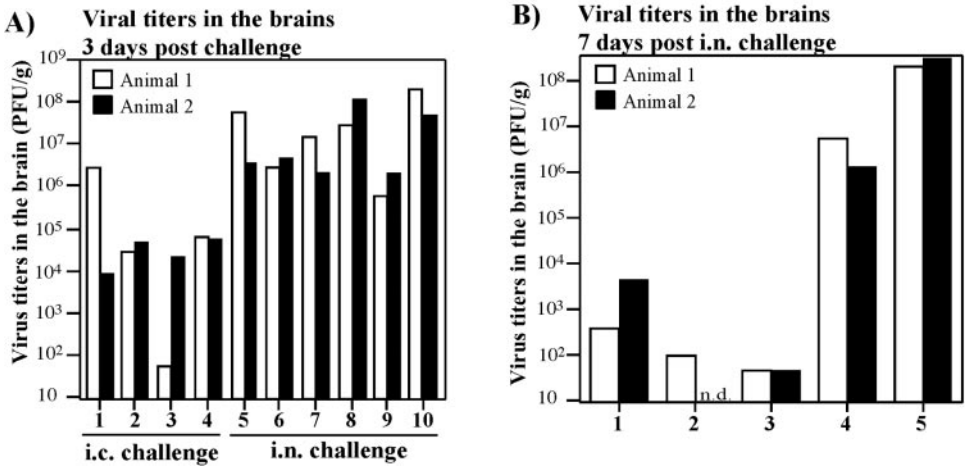

FIG. 5. Analysis of viral replication in brains of vaccinated and mock-vaccinated mice after i.c. or i.n. challenge with high doses of infectious ZPC738. Six-week-old female NIH Swiss mice were vaccinated with chimeric SIN/VEE viruses (5×10^5 PFU/animal via s.c. route) or PBS control (mock) and were subsequently challenged via i.c. or i.n. route with ZPC738 (2×10^5 PFU per animal in 20 μl of PBS). Two mice per group were euthanized, and brains were collected for analysis of viral titers. (A) Viral titers at 3 days postchallenge. Mice were vaccinated with (1) SAAR/TRD, (2) SIN/ZPC, (3) SIN/TRD, or (4) SIN83 and subsequently challenged 8 weeks later with ZPC738 via i.c. route into the left brain hemisphere; mice vaccinated with (5) SAAR/TRD, (6) SIN/ZPC, (7) SIN/TRD, (8) SIN83, (9) TC83, or (10) PBS alone (mock) were challenged via i.n. route. (B) Viral titers at 7 days postchallenge. Mice were vaccinated with (1) SAAR/TRD, (2) SIN/ZPC, (3) SIN/TRD, (4) SIN83, or (5) PBS alone (mock) and were subsequently challenged 8 weeks later via i.n. route with ZPC738. All viral titers were calculated in PFU per gram of tissue (n.d. = not detectable).

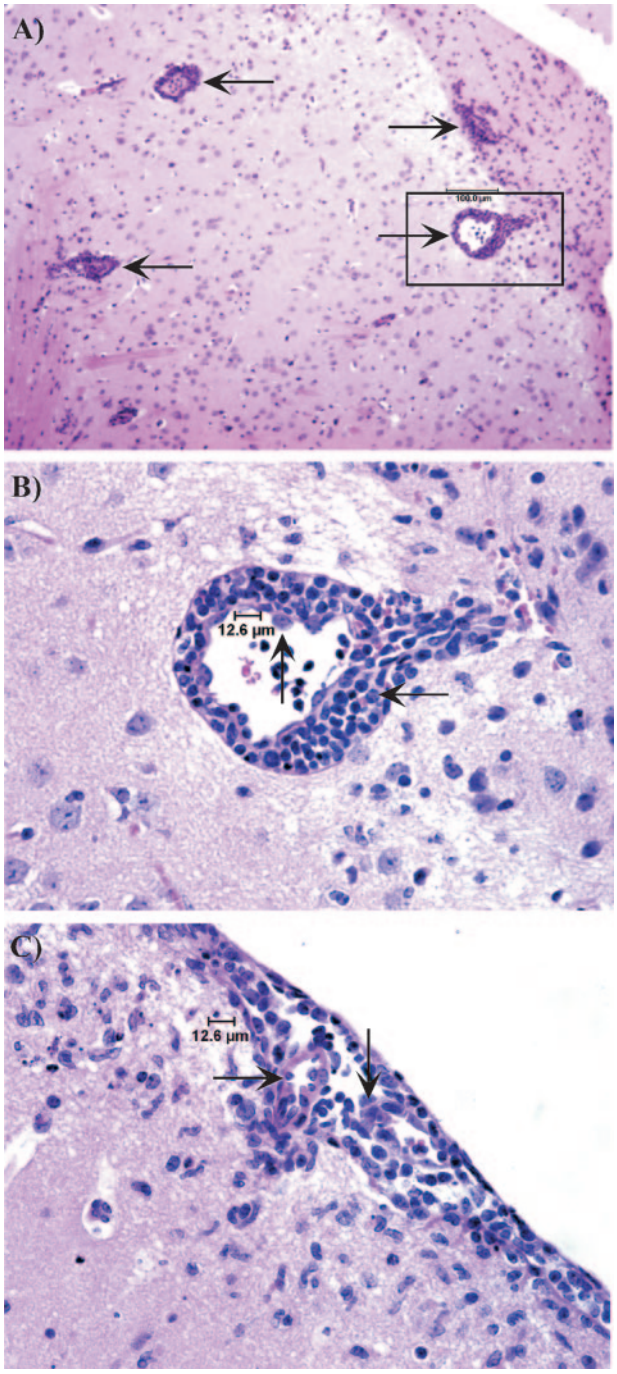

FIG. 6. Histopathological analysis of brains from vaccinated mice challenged i.n. with ZPC738. (A) Photomicrograph of H&E-stained brains obtained 3 days after infection. The horizontal arrows indicate the early inflammatory foci in the form of vascular (cuffing) and perivascular cellular infiltration in the brain stem. (B) Higher magnification image of that shown in panel A showing attachment of lymphocytes and monocytes to the vascular endothelium (vertical arrow) as well as infiltration of the vascular wall and the perivascular (Virchow-Robin) space (horizontal arrow). (C) Photomicrograph of H&E-stained brain obtained from the same animal. The classical acute meningitis is characterized by the expansion of meninges (arrows) by infiltration of inflammatory (mononuclear) cells.

the brains of mock-vaccinated animals. At this same time point, extensive neuronal cell death associated with neuronophagia was detected in mock-vaccinated animals but was absent in mice previously vaccinated with SIN/ZPC, SIN/TRD, SAAR/TRD, SIN83, or TC83. At 28 days postchallenge, the level of infectious virus and viral RNA in the brains of these vaccinated mice was below the limit of detection (data not shown). However, B and T cells were still demonstrable in all brains.

ZPC738 invades the brains even after rechallenge. In the above-described experiments, we found that vaccination with chimeric viruses, as well as with TC83, did not prevent virus replication in the brain following i.n. inoculation with ZPC738. To determine whether complete protection against infection is possible via i.n. route, we rechallenged SIN83-vaccinated (n = 6), SIN/TRD-vaccinated (n = 6), and SIN/ZPC-vaccinated (n = 6) mice at 28 days after the first challenge. Brains from the animals were collected on days 3, 7, and 28. We readily detected the presence of infectious ZPC738 in the brain on days 3 (two out of three animals) and 7 (one out of three animals) after repeat challenge; titers of infectious virus ranged from 50 to 1,000 PFU per gram of tissue. The titers of ZPC738 in the brain were dramatically lower than those measured in the first challenge experiment, with less inflammation observed (data not shown). The results of these experiments indicated that the first i.n. challenge with ZPC738 most likely induced a very strong immune response, but this response was still not sufficient to prevent reinfection of the brain.

Chimeric SIN/VEE viruses efficiently protect hamsters against VEEV infection. To further evaluate the safety of the designed chimeric viruses and to compare their immunogenicity with that of TC83, we performed additional experiments in a second animal model. Syrian golden hamsters were inoculated s.c. with 5×10^5 PFU of SIN/ZPC, SIN/TRD, SAAR/TRD, or TC83. No clinical disease was observed over a 3-week period p.i. with chimeric viruses. However, TC83-inoculated hamsters were hunched and lethargic on days 2 through 6 p.i.; one animal died at day 6 p.i. Hamsters in all groups (n = 3) developed detectable viremia after inoculation with the chimeric viruses as well as after inoculation with TC83 or ZPC738 (Fig. 8). The level of infectious virus in the blood was approximately 1,000-fold lower in animals infected with chimeric SIN/VEE viruses than in those infected with TC83 and was approximately 100,000-fold lower than in those infected with ZPC738 (Fig. 8). Immunized hamsters that were subsequently challenged with ZPC738 via s.c. inoculation remained disease free for 4 weeks, whereas mock-vaccinated hamsters succumbed to ZPC738 infection within 3 days. The results from these hamster studies indicate that these chimeric SIN/VEE viruses are safer than TC83. Additionally, the efficacy of these vaccine candidates is manifested by their ability to prevent the development of the VEEV-induced, shock-like disease in immunized hamsters.

DISCUSSION

Recent human VEEV epidemics and equine epizootics underscore the need for a safe and effective vaccine. The attenuated strains of VEEV, TC83 (developed in the United States) and 230 (developed in the former USSR), were developed by

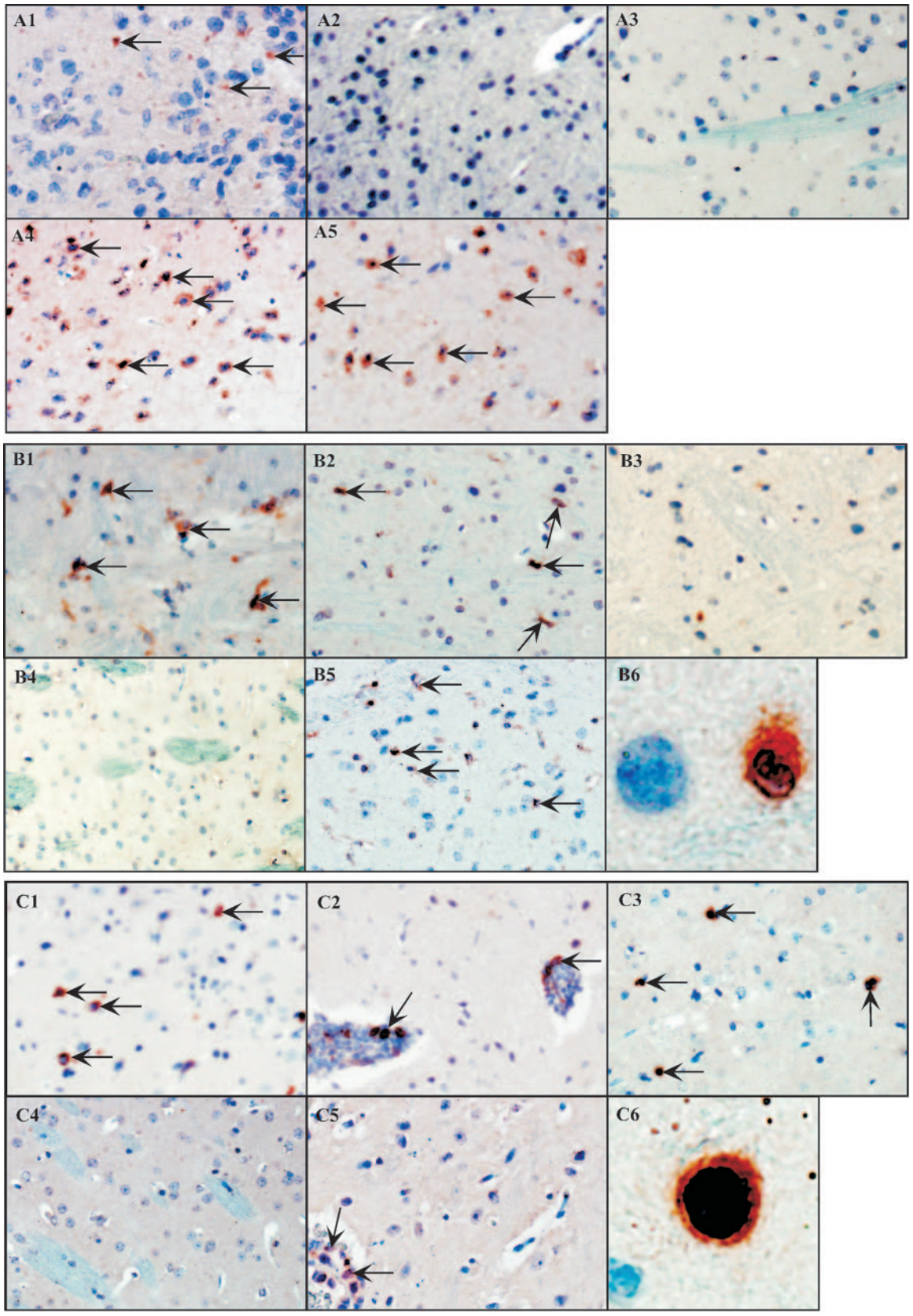

FIG. 7. Immunohistochemical analysis of VEEV antigen and T and B cells in brains from vaccinated and unvaccinated animals infected i.n. with ZPC738. Representative photomicrographs were obtained from 6-week-old female NIH Swiss mice that were vaccinated with SIN/ZPC via s.c. route and challenged via i.n. route with ZPC738 (2×10^5 PFU per animal in 20 μl of PBS) at 8 weeks postvaccination. Brains collected at the

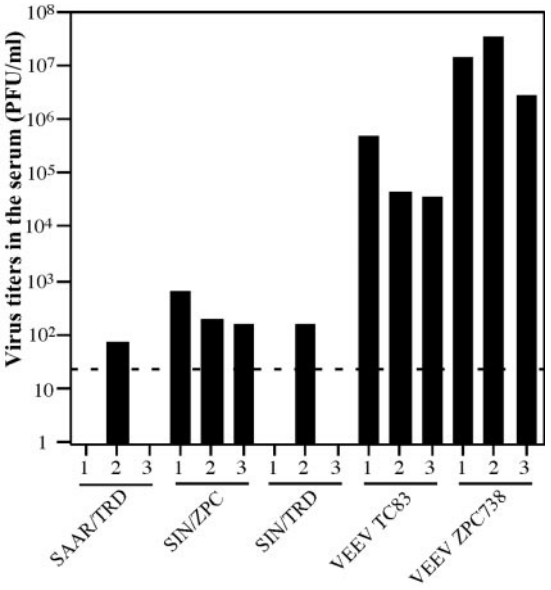

FIG. 8. Serum viremia in hamsters inoculated with SAAR/TRD, SIN/ZPC, SIN/TRD, TC83, and ZPC738. Three animals per group received 5×10^5 PFU of the indicated virus subcutaneously and were bled on the indicated days. Blood samples were collected via puncture of retroorbital plexus. A plaque assay was performed on BHK-21 monolayers. The final titer was estimated as PFU/ml serum. The dashed line presents the sensitivity limit of the plaque assay used.

serial passaging of the VEEV TRD in 83 passages in guinea pig heart cells (4) and 230 passages in embryonated eggs (11), respectively. The safety as well as the efficacy of both these vaccine strains is a concern. In particular, vaccination with TC83 is associated with adverse effects in 20 to 40% of vaccinated individuals (1, 6, 32). Furthermore, both TC83 and 230 strains have a number of mutations in the structural proteins, which despite contributing to attenuation, also change the antigenic structure and, potentially, the spectrum of antibodies induced by vaccination. TC83 has been used extensively for immunizing horses, and both strains have been used for decades to protect laboratory personnel working with VEEV. However, few human vaccines have been studied.

Utility of NIH Swiss mice as a model of VEE and for testing of vaccine candidates. One of the goals of our study was to further define the characteristics of NIH Swiss mice as a model of VEEV-induced encephalitis. Previous studies have demonstrated that the murine model is characterized by biphasic disease, which starts with the productive infection of lymphoid tissue and ends in the destruction of the CNS by viral replication and a "toxic" neuroinflammatory response (12, 13, 15, 16, 39, 42, 43). By the time encephalitis has developed in an infected mouse, the infectious virus is usually absent from the peripheral organs and blood (12, 13, 15, 16, 39, 42, 43). However, virus replicates to high titers in the brain, and mice die 5 to 7 days after infection due to fatal encephalitis, as previously demonstrated with ZPC738 (2, 31). Our results demonstrate that survival data may be insufficient for characterization of the efficacy of VEEV vaccines, especially if the vaccine is designed to provide protection against CNS infection. Moreover, they may not be applicable to VEEV, which is transmitted not only by mosquitoes but also by aerosol.

Genetic engineering of SINV chimeras for vaccine development and their safety. Recently, VEEV TRD was attenuated by using a gene engineering procedure in which a furin cleavage site deletion mutant was derived through site-directed mutagenesis (V3526) (7, 17), and it became a leading candidate in VEEV vaccine development (33). Although this new approach to the attenuation of alphaviruses is promising, the very high genetic plasticity and evolution rates of alphaviruses will always remain a concern (45, 49). Previously, we tested an alternative strategy to develop a live attenuated virus to induce efficient protection against VEEV. We designed a chimeric virus that contains the replicative machinery and *cis*-acting promoter elements from SINV and the structural genes derived from TC83. The SIN83 virus was more highly attenuated and protected mice against VEEV-caused lethal disease (31). However, we were concerned that in vivo, SIN83 might exhibit lower infectivity (as with TC83) and altered tissue tropism and induce a spectrum of VEEV-specific antibodies different from those induced by the structural proteins of the parental VEEV strains.

To evaluate their potential as attenuated vaccine candidates, we designed a spectrum of chimeric SIN/VEE viruses expressing VEEV structural proteins derived from VEEV TRD (epizootic IAB and C strains) and VEEV ZPC738 (enzootic ID strain) (2). In addition, one of the variants, SAAR/TRD, was designed to test whether specific mutations in the SINV backbone could increase the virulence or affect the highly attenuated phenotype of the chimeras.

Surprisingly, all of the chimeric viruses were attenuated in mice of various ages and both sexes and induced the production of VEEV-neutralizing antibodies. Chimeric viruses were not neuroinvasive and were incapable of establishing high lev-

following time points are presented: (1 and 4) 3 days postchallenge, (2 and 5) 7 days postchallenge, and (3) 28 days postchallenge. Immunohistochemical staining of brains for (A) VEEV antigens, (B) T cells, and (C) B cells are shown for vaccinated (1 to 3) and unvaccinated (4 to 5) mice following i.n. infection with ZPC738. Slides were counter-stained with Mayer's modified hematoxylin prior to mounting and microscopy. (A1 to 5) Localization of VEEV antigen in brains of vaccinated (A1 to 3) and mock-vaccinated (A3 to 4) mice. Viral antigen was detected in association with neuronal cells and neuropil in vaccinated animals (horizontal arrows) in the olfactory bulb on day 3 postchallenge (A1), while no viral antigen was detected on days 7 and 28 postchallenge (A2 and A3). Viral antigen was detected in brains of infected, unvaccinated animals in association with neuronal cells and neuropil in the brain cortex on day 3 (A4) and day 7 (A5). (B1 to 6) T cells in brains of vaccinated (B1 to 3, B6) and unvaccinated (B4 to 5) mice. CD3-positive cells (arrows) in brains of infected, vaccinated animals are shown for day 3 (B1), day 7 (B2), and day 28 (B3) postchallenge in the brain stem and in brains of infected, mock-vaccinated animals on day 3 (B4) and day 7 (B5). (B6) Higher magnification image of that shown in panel B3. (C1 to 6) B cells in brains of vaccinated (C1 to 3, C6) and mock-vaccinated (B4 to 5) mice. CD45R/B220-positive cells (arrows) in brains of infected, vaccinated animals on day 3 (C1), day 7 (C2), and day 28 (C3) postchallenge in the brain stem and in brains of infected, mock-vaccinated animals on day 3 (C4) and day 7 (C5) postchallenge. (C6) Higher magnification image of that shown in panel C3.

els of virus production in fully developed murine brains. We detected some minor differences in virulence between the chimeras only by infecting 6-day-old mice intracerebrally. According to the mortality rates and VEEV replication levels in the brains of mice, the viruses can be placed in the following order of virulence: ZPC738 ≫ TC83 ≫ SIN/ZPC > SAAR/TRD > SIN/TRD ≫ SIN83.

Protective effects of chimeric viruses. The final histopathological characteristics of the neuroinflammatory response were similar in immunized and mock-immunized animals following challenge with ZPC738 via either i.n. or i.c. route. However, we detected fundamentally different clinical outcomes in the animals, with the development of lethal encephalitis in mock-immunized animals that was absent in the immunized groups. Thus, we hypothesize that VEEV replication per se is not sufficient to cause overt clinical murine encephalitis and/or death. However, we cannot exclude the possibility that other clinical signs associated with viral infections occurred throughout the experiments, e.g., fever, weight loss, lymphopenia, and leukocytosis. We believe that VEEV pathogenesis, particularly the development of clinical encephalitis, depends on both virus-mediated and immune-mediated neuronal cell death. The combination of alphaviral replication and differential cytokine production causes lethal disease in neonatal mice in the absence of infiltration of the brain by the peripheral blood immune cells (52). In addition, SINV infection of neonatal mice results in a severe stress response, and attenuation correlates with reduced titers of serum and reduced titers of virus in the brain (51). Most studies have compared the pathogenesis of different alphaviruses (attenuated and nonattenuated strains) in naive animals, and little information is available about differential neuroinflammatory responses as described in this study. Development of encephalitic signs depends on two factors: direct damage to neurons and some specific characteristics of the neuroinflammatory response. The kinetics of neuroinflammation in immunized versus naive animals were fundamentally different, although we do not yet understand the biological importance of this difference.

Our results indicate that the early neuroinflammatory response in vaccinated animals is incapable of controlling the initial replication of the challenge virus in the brain, which occurs at levels comparable to those measured in naive animals on the first 3 days p.i. Production of infectious virus in the brain of a vaccinated mouse is reduced by day 7 and is undetectable by day 28. It is likely that the reduction of ZPC738 replication in the brain of vaccinated animals on day 7 might have an impact on survival; however, recent studies of genetically modified mice with preexisting anti-VEEV immunity have shown that high levels of replication in the brain over a time period of 12 days are not lethal (S. Paessler, unpublished data). It was previously demonstrated that host factors contribute to mortality in the neurovirulent Sindbis virus-induced encephalitis model for mice (50). Animals deficient in αβ but not γδ T cells had lower mortality rates when infected with neurovirulent Sindbis virus, indicating their different contribution to the outcome of the brain infection (38).

Comparative pathogenesis of chimeric SIN/VEE viruses in two animal models of VEE. Another objective of this study was to compare the safety and efficacy of chimeric viruses in NIH Swiss mice to those of chimeric viruses in Syrian golden hamsters, which are also susceptible to VEEV. In addition to the clinical disease manifestations and survival studies, we assessed the viremia levels of adult mice upon peripheral inoculation with chimeric viruses in comparison to those of hamsters. Peripheral VEEV infection in adult NIH Swiss mice and Syrian golden hamsters resulted in different disease patterns while it culminated in the same, uniformly lethal outcomes (18–20, 53). Infected hamsters develop prolonged lymphopenia and destruction of the reticuloendothelial system, resulting in bacterial endotoxemia/sepsis and shock-like death within 2 to 3 days without the development of encephalitis (19, 20, 53). Therefore, the Syrian golden hamster may represent a more stringent model than NIH Swiss mice to study the attenuation of VEEV-like viruses as well as the efficacy of vaccine candidates (19, 20, 53). In our experiments, hamsters were more susceptible to infection with chimeric viruses than mice. Hamsters developed transient, low-level viremia after inoculation with SIN/TRD, SIN/ZPC, or SAAR/TRD and prolonged, high-level viremia after infection with TC83 or ZPC738. After immunization with chimeras, hamsters did not show evidence of disease and were protected against infection following s.c. challenge with ZPC738.

The potential for improved safety and efficacy of VEEV vaccines. In summary, our results indicate that all of the chimeric SIN/VEE viruses that we tested are safe and immunogenic in adult mice and hamsters. They also induce protection against lethal encephalitis induced by ZPC738 infection in intranasal and intracerebral challenge models, as well as penetration of the CNS and clinical encephalitis after peripheral (s.c.) challenge in the murine model. Mock-vaccinated and vaccinated mice exhibit similar susceptibilities to ZPC738 replication in the brain in the first 3 days postchallenge that result in comparably high titers of virus in the brain. The neuroinflammatory response to ZPC738 infection in previously vaccinated mice is fulminant and rapid (present on day 3 postinfection). In contrast, the neuroinflammatory response of mock-vaccinated animals occurs later (absent on day 3 but present on day 7 postinfection) and induces uniformly lethal encephalitis in mice. Vaccinated animals that survive ZPC738 infection of the CNS and clear infectious virus from the brain are not completely protected against CNS reinvasion by ZPC738 in the subsequent second challenge, indicating the inability of ZPC738 to induce absolute protection in this challenge model.

Future directions. The Sindbis virus replicative machinery appears to play a critical role in attenuation of the chimeric viruses, offering the possibility of creating recombinant, Sindbis-based viruses expressing structural proteins of other VEEV subtypes, which can be manipulated under biosafety level 2 conditions for either vaccine development or potential diagnostic use (S. Paessler, unpublished).

Our future studies will be focused on the development of a highly efficient anti-VEEV neuroinflammatory response by attenuated chimeric SIN/VEE viruses in this animal model and its potential use to improve our current understanding of VEEV-induced encephalitis. In addition, ongoing experiments in our laboratory are testing the importance of T cells in the clearance of VEEV as well as in the death of preimmunized, challenged mice. The specific characteristics of VEEV-induced inflammation warrant further investigation, which may lead to the development of new therapeutic treatments.

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