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A chimeric Sindbis-based vaccine protects cynomolgus macaques against a lethal aerosol challenge of eastern equine encephalitis virus

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Abstract

Eastern equine encephalitis virus (EEEV) is a mosquito-borne alphavirus that causes sporadic, often fatal disease outbreaks in humans and equids, and is also a biological threat agent. Two chimeric vaccine candidates were constructed using a cDNA clone with a Sindbis virus (SINV) backbone and structural protein genes from either a North (SIN/NAEEEV) or South American (SIN/SAEEEV) strain of EEEV. The vaccine candidates were tested in a nonhuman primate (NHP) model of eastern equine encephalitis (EEE). Cynomolgus macaques were either sham-vaccinated, or vaccinated with a single dose of either SIN/NAEEEV or SIN/SAEEEV. After vaccination, animals were challenged by aerosol with a virulent North American strain of EEEV (NA EEEV). The SIN/NAEEEV vaccine provided significant protection, and most vaccinated animals survived EEEV challenge (82%) with little evidence of disease, whereas most SIN/SAEEEV-vaccinated (83%) and control (100%) animals died. Protected animals exhibited minimal changes in temperature and cardiovascular rhythm, whereas unprotected animals showed profound hyperthermia and changes in heart rate post-exposure. Acute inflammation and neuronal necrosis were consistent with EEEV-induced encephalitis in unprotected animals, whereas no encephalitis-related histopathologic changes were observed in the SIN/NAEEEV-vaccinated

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animals. These results demonstrate that the chimeric SIN/NAEEEV vaccine candidate protects against an aerosol EEEV exposure.

Keywords

Encephalitis; Aerosol; Nonhuman Primate; Alphavirus; Vaccine

1. Introduction

Eastern equine encephalitis virus (EEEV) is a positive-strand RNA virus in the genus *Alphavirus*, family *Togaviridae*. It occurs nearly throughout the Americas from southeastern Canada and the eastern U.S. to northern Argentina. North American EEEV (NA EEEV) strains are among the most virulent of all RNA viruses; human and equine case fatality rates usually exceed 50%, and human survivors typically experience permanent neurologic sequelae [1]. In Central and South America as in North America, EEEV strains are highly virulent for equids. However, human EEE has rarely been recognized in Latin America, probably due to the lower virulence for humans of the genetically and antigenically distinct strains there [2–4].

In addition to its importance as a mosquito-borne arboviral pathogen, EEEV is also a potent biological weapon [5] and a select agent and category B priority pathogen. There is no licensed therapeutic, and human immunization is limited to an investigational new drug (IND), formalin-inactivated vaccine [6,7] administered to laboratory personnel at risk.

We developed two novel, live-attenuated vaccine candidates that protect mice against fatal EEE [8]. These vaccine strains are chimeric alphaviruses, with the genetic backbone and nonstructural protein genes derived from the relatively benign Sindbis virus (SINV), and the structural protein genes derived from one of two wild-type (wt) EEEV strains: 1) a 1993 Florida strain isolated from mosquitoes, and; 2) a naturally attenuated, 1985 Brazilian mosquito strain that is avirulent for mice [9,10], hamsters (SCW, unpublished data), and marmosets [11]. Both chimeric viruses replicate efficiently in mammalian cell cultures and do not induce detectable murine disease or viremia following vaccination, but induce high titers of neutralizing antibodies and protect against fatal EEEV challenge.

Nonhuman primates (NHPs) have been instrumental to understanding the pathogenesis of encephalitic alphaviruses, as well as for evaluating vaccine candidates [12]. Cynomolgus macaques (*Macaca fascicularis*) have been the most widely used NHP model for human alphaviral pathogenesis, and develop disease similar to that described for humans. Furthermore, aerosol-challenge of NHP by alphaviruses has been established for biodefense purposes [5,13,14]. Therefore, we used the cynomolgus macaque to assess the safety and efficacy of our chimeric EEE vaccine candidates. Telemetry was also employed to sensitively measure sublethal disease.

2. Material and methods

2.1 Animals

Age- and sex-matched cynomolgus macaques (*Macaca fascicularis*) weighing 3–6 kg free of simian immunodeficiency virus (SIV), simian type D retrovirus (SRV), simian T-lymphotropic virus (STLV) and alphavirus antibodies [assayed by plaque reduction neutralization tests (PRNT)] were used. The study was approved by the Institutional Animal Care and Use Committee at Tulane University, and all animals were handled in accordance with guidance from the American Association for Accreditation of Laboratory Animal Care.

2.2 Vaccine and vaccination

Vaccine virus was rescued from chimeric alphavirus infectious cDNA clones as described previously [8]. In the first study, animals were vaccinated with SIN/NAEEEV (n=6), SIN/SAEEEV (n=6), or PBS diluent (n=4). In the second study, cohorts were vaccinated with either SIN/NAEEEV (n=5) or PBS (n=2). All animals were vaccinated subcutaneously (SC) in the upper deltoid with a single inoculation of either saline or 5.0 log₁₀ PFU of vaccine a volume of 100 µl and observed for signs of an adverse response after vaccination. In the first study, animals were bled on days 12, 24, and 45 after vaccination. For the second study, blood was collected on day 66 after vaccination. Following aerosol challenge, blood was also collected when the animals either succumbed to infection or 21 days after challenge when the experiment was terminated. For the first study, on day 12 after vaccination, telemetry devices were implanted and used to monitor heart rate (HRT) and body temperature (BWT) prior to and after aerosol challenge.

2.3 Radiotelemetry

Radiotelemetry transmitters with sensors capable of detecting biopotential signals of an electrocardiogram (ECG) (where signal amplitudes were recorded for HRT) as well as thermistor type sensors capable of detecting temperature signals [T31F-8b; Konigsberg Instruments (KI), Inc., Pasadena, CA] were surgically implanted in the abdomen. Cage-mounted antennas (TR38-1FG; KI, Inc.) were configured to receive and transmit signals to a data acquisition base station. Data collection was continuously recorded using a CA Recorder [Data Integrated Scientific Systems, Dexter, MI].

2.4 Serological assays

Neutralizing antibodies were assayed using 50% or 80% PRNT, and IgM/IgG responses were detected using an enzyme-linked immunosorbent assay (ELISA) as previously described [15]. For ELISAs, mouse brain antigens derived from NA EEEV strain NJ60, provided by the Centers for Disease Control and Prevention (CDC), Fort Collins, Colorado, were resuspended in 0.25 ml of water, then diluted 1:2000 in PBS. Then, 100 µl of diluted antigens were added to each well of a NUNC Immuno plate PolySorp 96-well plate (Nalgene Nunc International, Rochester, NY) and incubated at 4°C overnight. Plates were washed [PBS with 0.1% Tween 20 (Sigma)] and blocked [PBS, 0.1% Tween 20, 1% bovine serum albumin (BSA)]. Following the addition of 1:100 diluted sera, IgM and IgG were detected with either horseradish peroxidase (HRP)-conjugated alphavirus group-specific MAB 2A2C-3 (CDC) or IgG (NIH Nonhuman Primate Reagent Resource). Sera yielding absorbance values that exceeded the means of negative control sera by more than 2 standard deviations (0.1 ± 0.02) were considered positive.

2.5 Aerosol challenge

On either day 45 (study 1) or 66 (study 2) after vaccination, anesthetized macaques were challenged with virulent EEEV strain FL93-939 aerosols using a 16 liter head-only dynamic inhalation exposure system as described previously [16], and monitored for 21 days.

2.6 Gross and histopathological analyses

Necropsies were performed on animals when they succumbed to challenge or when the study was terminated 21 days later. Tissues were frozen for viral titration by plaque assay as well as placed into 10% zinc-formalin for histopathological analysis: mandibular lymph node, pharyngeal lymph node, tracheobronchial lymph node, mediastinal lymph node, lungs, heart, spleen, liver, kidney, adrenal gland, thyroid, brain, mesenteric lymph node, and testis or ovary. Sera and cerebrospinal fluids (CSF) were also tested for virus by plaque assay.

2.7 Statistical analysis

Statistical analysis was performed with SigmaPlot software, version 11.0 (Systat Software, Inc., San Jose, CA). Survival was analyzed using a 2-tailed Fishers exact test. Telemetric parameters were compared against preexposure values for each individual animal; any postexposure change 1.5 standard deviations divergent from the mean preexposure value were considered significant.

3. Results

3.1 Immunogenicity of SIN/EEEV vaccine candidates

Five of 6 macaques (83%) vaccinated with SIN/NAEEEV in the first study developed of neutralizing antibodies (Abs), first detected on day 12 post vaccination (Table 1). Animal ID67, which survived challenge, did not develop a detectable PRNT₈₀ response; however, a PRNT₅₀ titer of 1:160 was detected on day 45 after vaccination (data not shown). Overall, neutralizing Abs remained at initial levels or decreased slightly in the majority of the animals by day 24 or 45 post vaccination. Antibody responses in vaccinated animals from the second experiment, assayed on day 66 after vaccination, were slightly lower on average.

Despite having neutralizing Ab responses, two animals died after challenge: ID65 died on day 5, with PRNT₈₀ titers of 160–40 on days 12–45 post vaccination (Table 1) and ID80, with a PRNT₅₀ titer of 20 on day 66 and a negative PRNT₈₀ response (data not shown).

The SIN/SAEEEV vaccine strain was less immunogenic, with little to no neutralizing Ab development against NA EEEV (Table 2). Most animals in this cohort succumbed to infection by 4–5 days after challenge.

Following SIN/NAEEEV vaccination, most macaques developed IgM (5/6), which peaked on either day 12 or 24 post vaccination (Table 3). ID67, which survived challenge, was the only macaque that failed to develop an IgM response. In contrast, SIN/SAEEEV induced an IgM response in only one of 6 animals, not including the animal that survived challenge (ID70) (Table 4).

Most animals in the first study (8/11) developed an IgG response after vaccination with SIN/NAEEEV, peaking primarily on day 45 after vaccination (Table 5). Of the 3 animals that failed to develop a detectable IgG response, 2 survived challenge and developed IgG by the time of euthanasia. Following vaccination with SIN/SAEEEV, 5/6 animals developed IgG (Table 6), mostly peaking at day 24. However, these responses, based on OD values, were generally lower than those following SIN/NAEEEV vaccination (Table 5). The one animal that survived challenge (ID70) had the highest IgG titer prior to challenge.

3.2 Protection against EEEV challenge

The efficacies of the SIN/EEEV vaccines were assessed following aerosol EEEV challenge at approximately 7 (study 1) or 9 weeks (study 2) after a single vaccination. The individual doses of EEEV received in the challenge, estimated by plaque assay of the aerosol impinger samples collected during exposures, were $7.0 \pm 0.1 \log_{10}$ PFU (≈ 100 LD₅₀ of NA EEEV) [5]. The primary indicator of efficacy was protection from death (survival). Because this disease model was so acute and severe post exposure, it was impossible to obtain biosamples (including sera) from the exposed animals without undue stress caused by an anesthetic event. Therefore, only observation and remote telemetric monitoring (study one only) were performed post exposure.

The SIN/NAEEEV vaccine provided highly significant ($p=0.0023$) protection from fatal disease (figure 1), with 9 of the 11 animals (82%) surviving for 21 days until the study was

terminated. Animals receiving the SIN/SAEEEV were not significantly protected, with only one of the six (17%) surviving challenge. All (6/6) animals in the sham-vaccinated cohort died from encephalitis. The mean times of death for the SIN/SAEEEV and sham cohorts were 105 and 112 hr, respectively, which did not differ significantly.

Protective efficacy was also measured using the results of the telemetric monitoring through the challenge portion of the study. The parameters analyzed were core BWT (figures 2A, B, C), and HRT (figures 2D, E, F). Dramatic physiological changes occurred after EEEV infection of unvaccinated animals (figure 2). Animals in the SIN/SAEEEV- and sham-immunized groups experienced significant increases in core temperature (4/4 and 5/6, figures 2A, 2C, respectively) postexposure when compared to individualized pre-exposure values (data not shown). Only 1/6 animals in the NAEDEV group (figure 2B) experienced significant postexposure temperature change and this animal (ID65) succumbed to EEE. Similarly, significant changes in heart rate were observed in all SAEEEV- (6/6, figure 2D) and sham-immunized (4/4, figure 2F) animals. Significant changes in heart rate were also observed in the single animal (ID65) in the NAEDEV group (figure 2E) that was not protected from EEE.

3.4 Pathological changes associated with challenge

Gross and histopathologic lesions of the animals that succumbed to disease or were euthanized *in extremis* during the acute disease phase (days 3–5 post exposure) were consistent with the neurotropic nature of EEE [5]. Lesions in unprotected animals consisted of mild mononuclear cell infiltration of the meninges (figure 3A) and Virchow Robin spaces in the grey matter of the brain and spinal cord; neuronal necrosis with fragmentation and satellitosis (figure 3B); and neutrophilic infiltration of the grey matter and sometimes the meninges. Necrotic and hyaline neuron cell bodies were surrounded by mononuclear cells and neutrophils, but more often, neutrophils were found individually or in small clusters within the neuropiles. Some sections showed multifocal hemorrhage associated with fragmentation and vacuolization of the neuropiles and adjacent nerve fiber tracks (figure 3C). No lesions were found in the animals within the SIN/NAEEEV group that survived challenge.

No virus was detected by plaque assay in any of the SIN/NAEEEV vaccinated animals assayed 21 days after challenge (Table 7). The animals that succumbed to infection within 4–6 days of challenge had high viral loads in most organs. Although there was no detectable virus in the spleen and liver samples of SIN/SAEEEV-vaccinated animals, there was no evidence of an attenuated viral response in the central nervous system of these animals when compared to the controls.

4. Discussion

Although a variety of vaccines has been developed for EEE, including one used to protect laboratory workers under an IND permit [17], none has been tested for its ability to protect against aerosol challenge of primates. The chimeric SINV-based EEEV vaccines described here were previously shown protect mice from peripheral challenge [17], and here we describe their ability to protect NHPs from a lethal aerosol challenge. Eighty-two percent of animals challenged 45 or 66 days after a single vaccination survived, generally with strong neutralizing Ab responses and nearly complete protection against several physiological measures of disease.

Viral loads measured in the major organs, serum and CSF of animals that died 4–6 days after challenge revealed no major differences in titer, especially in the brain, based on vaccination status, emphasizing the importance of a robust early adaptive immune response,

especially for aerosol exposure. If this response is insufficient (and in one case, even in the presence of neutralizing antibodies), virus replicates to high titers in all major organs, apparently overwhelming the immune system. None of the vaccinated animals that survived challenge had detectable virus in the tissues or sera collected after euthanasia on day 21. To further document the efficacy of the SIN/NAEEEV vaccine, future experiments should include the collection of tissues six days after challenge, regardless of disease state, to determine protection against EEEV replication. Future studies should also determine whether cynomolgus macaques develop viremia following vaccination with SIN/NAEEEV and aerosol challenge with NA EEEV.

Our results contrast with those from a similar macaque study involving the closely related alphavirus, Venezuelan equine encephalitis (VEE) virus (VEEV) [18]. When cynomolgus macaques were vaccinated with a live attenuated (TC-83) or formalin-inactivated (C-84) VEE vaccine, 40% developed signs of disease after aerosol challenge.

Our findings emphasize the importance of testing vaccine efficacy using multiple animal models. Previous work in mice showed that both of our vaccine candidates induced protective immunity against a lethal challenge of NA EEEV [8]; however, the present study clearly indicates that SIN/SAEEEV does not protect against NA EEEV challenge in cynomolgus macaques via the aerosol route, presumably due to the limited cross-reactivity of antibodies developed in response to the SA envelope proteins against NA EEEV. The same previous mouse study [8] also suggested that SIN/NAEEEV was more immunogenic than SIN/SAEEEV, and our results corroborated this conclusion.

The dose of vaccine that was delivered to the macaques was the same used in the previous mouse study [8]; however, the resulting neutralization Ab titers were distinctly different. This suggests that the vaccine dose in the macaques was either too low to induce a robust neutralizing Ab response (and perhaps complete protection), or the immune response to these vaccine strains is host-dependent. Future work should include vaccinating macaques with higher doses of chimeric vaccine strains as well as attempting to boost the immune response with a second vaccination.

Surprisingly, the presence or absence of a neutralizing Ab response did not correlate completely with protection against fatal EEE. Similar findings were reported for cynomolgus macaques vaccinated with TC-83 and challenged with aerosolized VEEV [18]. A small percentage of human vaccinees typically fail to seroconvert following a single immunization with live-attenuated vaccines, including those for varicella [19] measles [20], hepatitis B [21] and VEEV strain TC-83 [22]. However, vaccinees who fail to seroconvert may be protected from subsequent exposure to virus, which can be associated with a virus-specific cell-mediated immune response [23]. Therefore, the administration of booster vaccinations is often recommended for non-responders to raise the level of neutralizing antibodies, which are generally strong correlates of protective immunity.

Similarly, our findings support the concept that outbred populations have a range of immunologic responses to vaccines and pathogens, where some individuals display a robust Ab response, and others a more robust T-cell response. Thus, it was not unexpected to see a range of protection in this outbred macaque model. The immune response of an outbred population is complex and depends on numerous variables among individuals. The specific mechanisms by which vaccines afford protection and make a good vaccine are still poorly understood [24].

Similar to the results of our mouse studies [8], neither SINV-based chimeric vaccine candidate caused overt signs of disease in macaques. Telemetry-based monitoring has been useful in evaluating other alphavirus vaccines in NHPs, including the VEEV vaccines,

TC-83 and C-84 [18]. They provide greater precision to detect physiological changes associated with vaccination and challenge, which can often be overlooked based simply upon clinical observation. After aerosol challenge with NA EEEV, the macaques vaccinated with SIN/NAEEEV showed very few physiological deviations from their pre-vaccination baseline status. Thus, these data were highly informative regarding the quality of immune protection.

Finally, another live-attenuated EEE vaccine, with attenuation based on the elimination of the EEEV subgenomic promoter and translation of the structural proteins via an internal ribosome entry site (IRES) from encephalomyocarditis virus [25] should be compared to SIN/EEEV in the cynomolgus macaque model. This vaccine candidate has greater environmental safety because it cannot infect mosquitoes, yet it appears to be equally safe, immunogenic and efficacious in the murine model.

In conclusion, the aerosol NHP model of EEE was recapitulated for use in this vaccine efficacy trial. Unprotected NHPs exhibited hallmarks of encephalitis described in prior modeling efforts. The majority of animals receiving the chimeric SINV-based vaccine possessing structural proteins of an NA EEEV variant (SIN/NAEEEV) were protected from death, and the disease severity associated with challenge was significantly reduced when compared to sham-vaccinated controls. However, unlike mice [8], our macaques vaccinated with the SA EEEV chimera (SIN/SAEEEV) showed little to no cross-protection when NA EEEV was used for challenge (<20% survival). Based on these findings, our SIN/NAEEEV vaccine candidate, the first live-attenuated EEE vaccine to show efficacy in primates, deserves further preclinical evaluation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- !! Chimeric vaccines were constructed from a cDNA clone containing genes from divergent EEE strains.
- !! The immunogenicity of each of the vaccines were assessed in cynomolgus macaques.
- !! All animals were challenged with a lethal aerosol dose of North American (NA) strain of the EEE virus.
- !! The NA-specific vaccine was protective; minimal cross reactive protection was observed.

Fig. 1. Survival of cynomolgus macaques vaccinated with either SIN/NAEEEV or SIN/SAEEEV and challenged with NA EEEV via the aerosol route. The control group included animals inoculated SC with saline. Statistical significance (*) was determined by 2-tailed Fishers exact test. $p=0.0023$.

Fig. 2. Physiological response of animals to EEE viral challenge. Core body temperature (A, B, C) and heart rate (D, E, F) for selected animals in the SIN/SAEEEV (A, D), SIN/NAEEEV (B, E) and sham-immunized controls (C, F). Values expressed are one hour means and all data have been truncated at 300 hours postexposure with time '0' representing time of viral aerosol exposure. Significance was determined by divergence of more than 1.5 standard deviations from the preexposure mean from individual animal.

Fig. 3.

Representative histologic lesions in the cerebrum of cynomolgus macaques following lethal aerosol challenge with NA EEEV. A-C) Temporal and frontal cortex of unprotected NHPs that died 4–5 days after challenge, 400x. A1) Mononuclear and polynuclear infiltrate in the meninges. A2) Inflammatory nodule in neuropile is composed of mainly polymorphonuclear leukocytes. B) Necrotic neurons (arrows) are surrounded by polymorphonuclear leukocytes and glial cells. C) Focal hemorrhage with inflammatory cells in an area of malacia. D) Frontal cortex of a NHP vaccinated with SIN/NAEEEV that survived the challenge infection, 200x.

Table 1Neutralizing antibody titers (PRNT₈₀) after vaccination with SIN/NAEEEV^a

Animal Number	Day 12 post vaccination	Day 24 post vaccination	Day 45 post vaccination	Day 66 post vaccination
ID63	80	80	20	n/a
ID64	320	320	160	n/a
ID65 ^b	160	160	40	n/a
ID66	320	80	320	n/a
ID67	<20	<20	<20	n/a
ID68	160	80	80	n/a
ID79	n/a	n/a	n/a	<20
ID80 ^b	n/a	n/a	n/a	<20
ID81	n/a	n/a	n/a	40
ID82	n/a	n/a	n/a	40
HV79	n/a	n/a	n/a	80
Number positive/total tested (percent positive)	5/6 (83)	5/6 (83)	5/6 (83)	3/5 (60)

^a NA EEEV strain FL93-939 was used to determine the neutralizing antibody titers. n/a, sample not available.

^b Animals ID65 and ID80 died on days 5 and 6 post challenge, respectively, while the remaining animals in the cohort survived aerosol challenge with NA EEEV strain FL93-939.

Table 2Neutralizing antibody titers (PRNT₈₀) after vaccination with SIN/SAEEEV^a

Animal Number	Day 12 post vaccination	Day 24 post vaccination	Day 45 post vaccination
ID69	20	20	<20
ID70 ^b	<20	20	20
ID71	40	40	<20
ID72	<20	40	40
ID73	40	<20	80
ID74	<20	<20	<20
Number positive/total tested (percent positive)	3/6 (50)	4/6 (67)	3/6 (50)

^aSA EEEV strain BeAr436087 was used to determine the neutralizing antibody titers.^bAnimal ID70 survived aerosol challenge, while the remaining animals succumbed 4–5 days after challenge.

Table 3IgM response after vaccination with SIN/NAEEEV and aerosol challenge with NA EEEV strain FL93-939^a

Animal Number	Day 12 post vaccination	Day 24 post vaccination	Day 24 post Day 45 or 66 post vaccination	Day 21 post challenge
ID63	0.40	0.21	0.05	0.03
ID64	0.92	1.19	0.48	0.28
ID65	1.03	0.94	0.27	0.09 ^b
ID66	1.12	1.12	0.21	0.06
ID67	0.00	0.07	0.00	0.03
ID68	0.34	0.42	0.15	0.19
ID79	n/a	n/a	0.01	0.01
ID80	n/a	n/a	0.00	0.00 ^c
ID81	n/a	n/a	0.02	0.04
ID82	n/a	n/a	0.02	0.02
HV79	n/a	n/a	0.01	0.01

^aOD values >0.2 are considered positive (in bold); serum was diluted at 1:100 for detection, n/a, sample not available.^bSerum sample was collected on day 5 post challenge when the animal died.^cSerum sample was collected on day 6 post challenge when the animal died.