A DNA Vaccine for Venezuelan Equine Encephalitis Virus Delivered by Intramuscular Electroporation Elicits High Levels of Neutralizing Antibodies in Multiple Animal Models and Provides Protective Immunity to Mice and Nonhuman Primates

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We evaluated the immunogenicity and protective efficacy of a DNA vaccine expressing codon-optimized envelope glycoprotein genes of Venezuelan equine encephalitis virus (VEEV) when delivered by intramuscular electroporation. Mice vaccinated with the DNA vaccine developed robust VEEV-neutralizing antibody responses that were comparable to those observed after administration of the live-attenuated VEEV vaccine TC-83 and were completely protected from a lethal aerosol VEEV challenge. The DNA vaccine also elicited strong neutralizing antibody responses in rabbits that persisted at high levels for at least 6 months and could be boosted by a single additional electroporation administration of the DNA performed approximately 6 months after the initial vaccinations. Cynomolgus macaques that received the vaccine by intramuscular electroporation developed substantial neutralizing antibody responses and after an aerosol challenge had no detectable serum viremia and had reduced febrile reactions, lymphopenia, and clinical signs of disease compared to those of negative-control macaques. Taken together, our results demonstrate that this DNA vaccine provides a potent means of protecting against VEEV infections and represents an attractive candidate for further development.

Venezuelan equine encephalitis virus (VEEV) is a nonsegmented, positive-sense RNA virus of the genus Alphavirus in the family Togaviridae (17). Naturally transmitted by mosquitoes through rodent hosts, VEEV is highly pathogenic for equines and humans and has sporadically caused widespread epidemics in North, Central, and South America (48). While most equine and human outbreaks have been caused by the epizootic IAB and IC subtypes of VEEV, isolated cases of disease caused in equines and humans by infection with the enzootic ID and IE subtypes have also been reported (10, 32, 50). Regardless of the variety, human infection with VEEV typically results in an acute, incapacitating, but self-limiting disease characterized by fever, headache, lymphopenia, myalgia, and malaise (3). Severe neurological disease, including fatal encephalitis, can also result from VEEV infection of humans, although the case fatality rate is estimated to be low (≤1%) (45). However, numerous documented laboratory accidents and the results of animal studies have demonstrated that VEEV is also highly infectious in aerosols, and infection with aerosolized VEEV could potentially result in higher mortality than that observed with natural infection (15, 18, 26). In addition to producing incapacitating or lethal infections and being infectious in aerosols, VEEV is also easily grown to high titers in inexpensive and unsophisticated cell culture systems and is relatively stable (45). As a result, VEEV represents a significant potential biological defense threat and is classified as a category B priority biodefense agent by both the Centers for Disease Control and Prevention and the National Institute of Allergy and Infectious Diseases.

There are no licensed human vaccines for VEEV. While live-attenuated and formalin-inactivated VEEV vaccines are currently being utilized under Investigational New Drug (IND) status to protect laboratory workers and other at-risk personnel against VEEV, these vaccines have significant safety and immunogenicity shortcomings. A live-attenuated vaccine for VEEV, TC-83, provides long-lasting immunity and protection from both subcutaneous and aerosol VEEV challenges; however, it causes adverse reactions in approximately 25% of recipients, and approximately 20% of recipients fail to develop a detectable immune response (31, 36). A formalin-inactivated VEEV vaccine derived from TC-83, C-84, is well tolerated, but it requires frequent boosting to elicit detectable immune responses and provides poor protection against a VEEV aerosol challenge (8, 24). Due to the significant limitations associated with these existing investigational VEEV vaccines, the development of improved vaccines that can safely and effectively protect against VEEV infections in humans is needed.

Next-generation VEEV vaccines, including live-attenuated, inactivated, attenuated Sindbis/VEEV chimeric viruses, alphavirus replicons, and DNA vaccines, are all currently at various stages of development (33). Genetic vaccination with DNA...
plasmids expressing immunogenic proteins has numerous inherent advantages as a platform for the development of next-generation vaccines. Among the benefits of this method are that DNA vaccines can be rapidly and cost-effectively produced without the need to propagate a pathogen, do not require the inactivation of infectious organisms, avoid problems of preexisting or vector-induced immunity due to the lack of a host immune response to the plasmid backbone, and have exhibited a favorable safety profile in numerous human clinical trials (13).

In previous studies performed in our laboratory, mice vaccinated with a DNA vaccine expressing the structural proteins (C-E3-E2-6K-E1) of VEEV IAB (strain Trinidad donkey) by particle-mediated epidermal delivery (PMED) or “gene gun” developed strong overall antibody responses against VEEV IAB; however, the VEEV-neutralizing antibody responses were low and only 80% protection against a lethal aerosol challenge was observed (42). Cynomolgus macaques vaccinated with this VEEV DNA vaccine by PMED developed detectable levels of VEEV IAB-neutralizing antibodies, but only partial protection was observed upon aerosol challenge (12). As our ultimate goal is to develop an effective human vaccine for encephalitic alphaviruses, we subsequently tested directed molecular evolution or “gene shuffling” of the envelope protein genes as a means to improve the neutralizing antibody response to VEEV and eastern equine encephalitis virus (EEEV) and western equine encephalitis virus (WEEV) DNA vaccines. DNA vaccines expressing representative variants from a library in which the E2 envelope glycoprotein genes of five parent viruses (VEEV subtypes IAB and IE, Mucambo virus, EEEV, and WEEV) were recombined and the E1 envelope glycoprotein gene of VEEV IAB was held constant elicited VEEV IAB-neutralizing antibody titers significantly higher than those obtained with the wild-type parent DNA vaccine and provided improved protection against an aerosol VEEV IAB challenge (11). While promising, the in vitro gene recombination was technically difficult, the screening of variants for improved immunogenicity was labor-intensive, and in our initial attempts, we did not identify any variant envelope glycoprotein vaccines that offered improved immunogenicity against EEEV and WEEV. Consequently, we have continued to investigate a variety of approaches for improving the immunogenicity and protective efficacy of encephalitic alphavirus DNA vaccines to include optimization of the constructs and evaluating alternative delivery methods. Here, we report the results of our studies with a codon-optimized VEEV DNA vaccine construct delivered by intramuscular (i.m.) electroporation (EP).

MATERIALS AND METHODS

Vaccines. The wild-type VEEV plasmid DNA vaccine (VEEVp) was constructed by inserting the cloned 26S structural genes (C-E3-E2-6K-E1) of VEEV IAB strain Trinidad donkey (GenBank accession number L01442) behind the cytomegalovirus immediate-early promoter of the eukaryotic expression vector pWGR7077 as previously described (42). Codon-optimized VEEV IAB structural genes with the capsid gene (VEEVpC) and lacking the capsid gene (VEEVpCO) were generated by subjecting the sequences to the GeneOptimizer bioinformatic algorithm, followed by synthesis of the codon-optimized genes (Geneart, Regensburg, Germany). Plasmid DNA vaccines were constructed by cloning the synthesized codon-optimized genes into the NotI and BglII restriction sites of pWGR7077. The plasmids used in these studies were manufactured by Aldevron (Fargo, ND). The live-attenuated VEEV vaccine TC-83 was manufactured by the National Drug Company (Philadelphia, PA).

Animals and vaccinations. Female BALB/c mice (6 to 8 weeks old) were vaccinated with plasmid DNA diluted to the appropriate concentration in calcium- and magnesium-free phosphate-buffered saline (Invitrogen) by i.m. EP using the TriGrid Delivery System (TDS; Ichor Medical Systems, San Diego, CA) as described previously (30). Briefly, mice anesthetized by i.m. injection of a diluted acepromazine-ketamine-xylazine mixture or with isoflurane gas were injected in one tibialis anterior muscle with 20 μl of a DNA solution using a 310-μm U-100 insulin syringe (Becton Dickinson) inserted into the center of a TriGrid electrode array with 2.5-mm electrode spacing. Injection of DNA was followed immediately by electrical stimulation at an amplitude of 250 V/cm, and the total duration was 40 ms over a 400-ms interval. Some control vaccinations consisted of i.m. injection of the DNA solution as described above without the application of electrical stimulation. The live-attenuated VEEV IND vaccine TC-83 was delivered by subcutaneous injection.

Female New Zealand White rabbits (3 to 3.5 kg) were anesthetized with isoflurane gas and vaccinated with plasmid DNA diluted to the appropriate concentration by i.m. EP with the Ichor TDS essentially as described above for mice. However, rabbits were injected with 0.5 ml of DNA solution in one quadriceps muscle with a 1-cm2 syringe (Becton Dickinson) with a 23-gauge needle. A TriGrid electrode array with 6.0-mm electrode spacing was used. Healthy adult male cynomolgus macaques (≥5 kg) obtained from the nonhuman primate colony at USAMRIID were screened for the presence of serum neutralizing antibodies against VEEV IAB, VEEV IE, VEEV IIIA, EEEV, and WEEV by plaque reduction neutralization tests (PRNT) as described below before being assigned to this study. Macaques were anesthetized by i.m. injection of Telazol and vaccinated with plasmid DNA diluted to the appropriate concentration by i.m. EP with the Ichor TDS as described above for rabbits.

All animal research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals (23). The USAMRIID facility where this animal research was conducted is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Immunological assays. At various times after vaccination, as described later in the text and shown in the figures, blood samples were collected from anesthetized mice by retro-orbital bleeding, from anesthetized rabbits by central auricular artery bleeding, and from anesthetized macaques by femoral vein bleeding, and serum was recovered by centrifugation. Total IgG anti-VEEV endpoint antibody titers were determined for serum samples by standard enzyme-linked immunosorbent (ELISA) using sucrose-purified, irradiated whole VEEV IAB antigen as described previously (21). Briefly, 2-fold serial dilutions of sera starting at 1:100 were incubated with 250 ng per well of antigen in 96-well plates. Horseradish peroxidase (HPR)-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibodies (Sigma-Aldrich) and 2,2′-azinobis(3-ethylbenzthiazoline-sulfonic acid) (ABTS) peroxidase substrate (KPL, Gaithersburg, MD) were used for detection. For antibody titrating ELISAs, HRP-conjugated anti-mouse IgG and anti-rabbit IgG secondary antibodies (Bethyl Laboratories, Montgomery, TX) were used. The optical density at 405 nm was determined using a SpectraMax M2e microplate reader (Molecular Devices, Sunnyvale, CA), and the endpoint titers were calculated using Softmax Pro v5 (Molecular Devices).

The VEEV IAB-neutralizing antibody titers were determined for serum samples by PRNT as described previously (21). Briefly, 2-fold serial dilutions of sera were mixed with equal volumes of medium containing 200 FFU of virus and incubated for 24 h at 4°C. The virus-antibody mixtures were then used to infect confluent monolayers of Vero cells contained in 6-well plates for 1 h at 37°C, after which an overlay consisting of 0.6% agar in complete EBME (Invitrogen) without phenol red was added. The plates were stained 24 h later by the addition of an overlay containing 5% neutral red and 0.6% agar in complete EBME without phenol red, and the plaques were counted 24 h after staining. The antibody titer required for an 80% reduction of the number of plaques (PRNT80) was then calculated for each serum sample.

Anti-VEEV cellular immune responses were analyzed by gamma interferon (IFN-γ) enzyme-linked immunosorbent (ELISPOT) assay using standard methods as described previously (46). Briefly, splenocytes isolated from individual spleens obtained from vaccinated mice using BD Falcon 100-μm nylon cell strainers (Becton Dickinson) were resuspended in complete RPMI 1640 medium (Mediatech, Manassas, VA). The resuspended splenocytes from each spleen were then added at a concentration of 2 × 106 cells per well to triplicate wells of MultiScreen HTS IP 0.45-μm polycarbonate filter 96-well plates (Millipore, Billerica, MA) previously coated with mouse IFN-γ ELISPOT assay capture antibody (Becton Dickinson). The splenocytes were then cultured with no pep-
tide, 10 μg/ml concanavalin A (Sigma-Aldrich), 20 μg/ml β-galactosidase peptide TPHPARIGL (New England Peptide, Gardner, MA), or 10 μg/ml pooled 15-mer peptides with an 11-base overlap spanning the VEEV IAB E2 or E1 envelope glycoprotein (Pepsan, Leystad, Netherlands) for 24 h at 37°C with 5% CO₂. Secreted IFN-γ ELISPOT assay detection antibody (Becton Dickinson), for 1 h at room temperature with mouse IFN-γ ELISPOT assay detection antibody (Becton Dickinson), and for 20 min at room temperature with AEC substrate (Becton Dickinson). The substrate reaction was then stopped by washing the plates with deionized H₂O, the plates were dried for 2 h at room temperature, and the spots were enumerated.

Aerosol challenge of mice. Mice were placed into a class III biological safety cabinet located inside a biosafety level 3 containment suite and exposed in a whole-body aerosol chamber to a VEEV aerosol created by a Collison nebulizer for 10 min as previously described (20). VEEV IAB (strain Trinidad donkey) was diluted to an appropriate starting concentration in Hanks’ balanced salt solution (HBSS) containing 1% fetal bovine serum for use in aerosol generation. Samples collected from the all-glass impinger (AGI) attached to the aerosol chamber were analyzed by plaque assay on Vero cells using standard methods as previously described to determine the inhaled dose of VEEV (38). The mice were monitored twice daily for clinical signs of illness and death for 28 days postchallenge, and any moribund animals were euthanized. After the postchallenge observation period was completed, Kaplan-Meier survival analysis of the protection data was performed.

Aerosol challenge of macaques. Before aerosol exposure, macaques were anesthetized by i.m. injection of 6 mg/kg Telazol and a whole-body plethysmograph was taken for 3 min to determine the respiratory capacity of the animal. The macaques were then placed into a class III biological safety cabinet located inside a biosafety level 3 containment suite and exposed in a head-only aerosol chamber to a VEEV aerosol created by a Collison nebulizer for 10 min as previously described (38, 40). VEEV IAB (strain Trinidad donkey) was diluted to an appropriate starting concentration in HBSS containing 1% fetal bovine serum for use in aerosol generation. Samples collected from the all-glass impinger (AGI) attached to the aerosol chamber were analyzed by plaque assay on Vero cells using standard methods as described above to determine the inhaled dose of VEEV. The macaques were monitored at least twice daily for clinical signs of illness for 28 days postchallenge. The macaques were observed for neurological signs of infection, changes in activity and behavior, and responses to stimuli using predetermined criteria. The observers were blinded and were not aware of which macaques belonged to which groups.

On days 3 to 1 relative to aerosol exposure and for 7 days postchallenge, the macaques were anesthetized with 3 mg/kg Telazol and blood samples were collected to assess viremia and lymphopenia. Serum viremia was measured by plaque assay as described above. Complete blood counts were determined with a Coulter T series instrument (Beckman Coulter, Brea, CA) and a manual differential count.

TA10TA-D700 radiotelemetry implants (Data Sciences International, St. Paul, MN) were surgically implanted subcutaneously on the dorsal surface, and macaques were allowed ≥30 days to recover from surgery and acclimate before VEEV exposure. Body temperatures were recorded every 15 min by using the DataQuest A.R.T. 2.1 System (Data Sciences International). Temperature monitoring was initiated 14 days before VEEV exposure to develop baseline temperature data to fit an autoregressive integrated moving average model as previously described (38, 40). Forecast values for the postchallenge time periods were based on the training model extrapolated forward in time. Residual temperature changes after exposure were determined by subtracting the predicted temperature from the actual temperature recorded for each point.

Statistical analysis. Log₁₀ transformations were applied to whole-virus ELISA titers and PRNTₜ₀ values. Mixed-model analysis of variance with Tukey’s post hoc tests was used to compare titers between groups at each time point. Kaplan-Meier survival analysis was performed for the mouse challenge study data, with log rank tests used for comparison of survival curves. The effects of whole-virus ELISA titers and PRNTₜ₀ on the probability of survival were assessed using a backward selection logistic regression model. Analyses were conducted using SAS v9.2 (SAS Institute, Cary, NC).

RESULTS

Vaccine design. In our previous studies, we demonstrated that a DNA vaccine expressing the wild-type structural proteins of VEEV IAB elicited partial protective immunity against a homologous viral aerosol challenge when administered by PMED in macaques (12). In an attempt to rationally design an improved VEEV DNA vaccine, we generated a construct expressing codon-optimized envelope glycoprotein genes. This approach was pursued because rational alterations in codon usage have been shown in numerous studies to have the potential to increase the expression of exogenous genes (7, 9, 47). Moreover, a codon-optimized adenovirus-based VEEV vaccine was recently reported to have improved immunogenicity and protective efficacy in mice (51). We also eliminated the capsid gene from this vaccine because earlier studies by others indicated that the capsid proteins of VEEV and EEEV can be cytotoxic and can inhibit cellular transcription and nuclear import in vertebrate cells (1, 2, 16). The final construct (VEEVₜₐ) included the E3-E2-6K-E1 coding region of VEEV IAB, strain Trinidad donkey, which was codon optimized to reflect the codon bias of Homo sapiens genes and to avoid negatively cis-acting motifs in the genes that can reduce expression.

Immunogenicity in mice. The results of studies performed in our laboratory and by others have demonstrated that i.m. EP is a highly effective method for facilitating the uptake and expression of DNA vaccines in multiple animal species (27–29, 44). In addition, i.m. EP has been used to deliver DNA vaccines in several clinical trials, where it has shown promise for eliciting immune responses against a variety of antigens (49). Therefore, we evaluated this method of delivery with our VEEVₜₐ DNA vaccine in mice. Groups of six female BALB/c mice were vaccinated with i.m. EP three times at 3-week intervals with 25 μg, 5 μg, or 1 μg of the VEEVₜₐ DNA vaccine or, for comparison, with the vaccine used in our earlier studies, which expresses the capsid gene, as well as the envelope protein genes of wild-type VEEV (VEEVₜₐ). A negative-control group received the plasmid vector with no insert.

Serum samples obtained 3 weeks after each vaccination were assayed for total anti-VEEV IgG antibodies by ELISA and for VEEV-neutralizing antibodies by PRNT. The mean ELISA titers of mice vaccinated with the VEEVₜₐ plasmid DNA were significantly higher than those of mice vaccinated with the VEEVₜₐ construct for the 5-μg dose groups after a single vaccination and for the 1-μg dose groups after each of the three vaccinations (Fig. 1A). After two or three vaccinations with the 5-μg dose and after each of the three vaccinations with the 25-μg dose, the mice that received these two plasmids displayed similar mean ELISA titers (P > 0.05). Within each group, there was no significant increase in the mean titers observed after two or three vaccinations.

The mean PRNTₜ₀ titer elicited in mice vaccinated with the VEEVₜₐ DNA was significantly higher than that observed in mice vaccinated with the VEEVₜₐ construct for all dose groups at all days (P < 0.01 to 0.0001) (Fig. 1B). Interestingly, the mean PRNTₜ₀ titer of mice vaccinated with 1 μg of the VEEVₜₐ DNA was higher than that of mice that received 25 μg of the VEEVₜₐ construct after each of the three vaccinations. As observed for the total IgG antibody titers, there was only a small difference in the level of antibodies measured by PRNT after two or three vaccinations.

To directly assess the effect of EP, a separate experiment was conducted in which groups of six female BALB/c mice were vaccinated twice at a 3-week interval with 5 μg of the VEEVₜₐ DNA vaccine delivered by i.m. injection with and
without EP. After the second vaccination, both the mean ELISA and PRNT antibody titers were significantly higher in mice that received the vaccine with EP than in those that received it by injection only (Fig. 2).

Comparison of the immunogenicity and protective efficacy of codon-optimized VEEV DNA vaccines with and without the capsid protein. As a first step toward assessing the protective efficacy of the VEEVCO DNA vaccine delivered by i.m. EP, a challenge study was performed with mice. In this study, the potential contribution of the capsid protein was also examined by including a codon-optimized construct expressing all of the structural proteins of VEEV, including the capsid protein (VEEVCCAP). Groups of 10 female BALB/c mice were vaccinated twice at a 3-week interval with 5 μg of the VEEVCO DNA vaccine delivered by i.m. EP. All mice received the empty vector as a negative control. A positive-control group was included in this experiment in which the mice were given a single subcutaneous injection of live-attenuated VEEV IND vaccine TC-83 (1 × 10⁷ PFU).

There was no statistically significant difference in the mean ELISA titers observed between serum samples collected 3 weeks after one or two vaccinations with the VEEVCO or VEEVCCAP construct; however, within both of these groups, the second vaccination significantly boosted the antibody titer compared to that measured after one vaccination (Fig. 3A). Comparing the overall antibody responses elicited by the DNA vaccines to that resulting from vaccination with TC-83 revealed that the VEEVCO DNA vaccine and TC-83 elicited similar titers after a single vaccination, whereas two vaccinations with VEEVCCAP were required to match that obtained with TC-83. While the mean neutralizing antibody titers of mice that received two vaccinations with the VEEVCO plasmid were similar to those of mice vaccinated with TC-83, those of mice that received two vaccinations with the VEEVCCAP DNA construct remained significantly lower than those vaccinated with TC-83 (Fig. 3B).

To assess vaccine efficacy, the mice from all groups were challenged 4 weeks after the final vaccination with 10⁴ PFU (≈1,000 50% lethal doses [LD₅₀]) of VEEV IAB strain Trinidad donkey administered by the aerosol route. Control mice vaccinated with the empty vector all displayed signs of illness after the challenge, including ruffled fur, loss of appetite, inactivity, and hunched backs, and all died or were euthanized due to morbidity (Fig. 4). Consistent with results from previous aerosol challenge studies (11, 42), 90% of the mice that received TC-83 survived the challenge and the single mouse that did not survive the challenge did not respond to the vaccine.
elicit cell-mediated immune responses, we measured IgG antibody subtypes in pooled sera obtained from mice in the challenge study after their second vaccination. As seen previously (42), mice vaccinated with TC-83 had a preponderance of IgG2a antibodies associated with a predicted Th1 skew in the immune response (Fig. 5A). In contrast, mice vaccinated with the VEEV CO DNA vaccine delivered by i.m. EP had comparable IgG1 and IgG2a antibody titers indicative of a more balanced Th1/Th2 immune response.

To directly evaluate the possibility that the VEEV CO DNA vaccine could elicit cell-mediated immune responses, groups of female BALB/c mice (n = 6) were vaccinated twice at a 3-week interval with 5 μg of the empty vector or VEEV CO plasmid DNA delivered by i.m. EP. Two weeks after the second vaccination, splenocytes isolated from the vaccinated mice were restimulated with no peptide, concanavalin A, a β-galactosidase peptide, or pools of overlapping peptides spanning the VEEV IAB E2 or E1 envelope glycoprotein and analyzed by IFN-γ ELISPOT assay. Splenocytes restimulated with concanavalin A produced spots that were too numerous to count (data not shown). Although splenocytes restimulated with no peptide or with the β-galactosidase peptide failed to produce a response in this assay, those restimulated with pooled peptides representing the VEEV E2 or E1 protein produced measurable IFN-γ responses (Fig. 5B). Interestingly, the mean IFN-γ response of splenocytes restimulated with the VEEV E2 pooled peptides was significantly higher than that of those restimulated with the VEEV E1 pooled peptides.

**Durability of the antibody response to VEEV CO in rabbits.** Although rabbits are not a challenge model for VEEV, their large muscle size permits EP delivery of higher doses of DNA plasmids that are similar to those expected to be delivered to humans, and they are a useful model for studying antibody durability after vaccination. To assess the durability of the antibody response to VEEV CO, we vaccinated each of 5 female New Zealand White rabbits with 500 μg of the VEEV CO DNA vaccine delivered by i.m. EP on days 0, 28, and 230 and measured their antibody responses to VEEV on days 0, 28, 42, 230,
The rabbits produced high titers of VEEV-neutralizing antibodies after a single vaccination with the VEEVCO DNA construct, and upon a second vaccination, their mean PRNT80 titer was significantly boosted (Fig. 6). The PRNT80 titer declined between days 42 and 230 yet remained remarkably high, with a mean of 1:1,000 at day 230. In addition, the mean PRNT80 titer was significantly boosted with an additional DNA vaccination performed on day 230, and the level of VEEV-neutralizing antibodies observed on day 266 was not significantly different from that observed on day 42.

Immunogenicity and protective efficacy of the VEEVCO DNA vaccine in cynomolgus macaques. Nonhuman primates have been used extensively for aerosol challenge studies with VEEV, and this animal model reflects many aspects of human disease caused by VEEV infection (40). We assessed the immunogenicity and protective efficacy of the VEEVCO DNA vaccine in an established model of VEEV infection of nonhuman primates that has been refined by the use of telemetry to evaluate fever responses (38). Groups of four adult male cynomolgus macaques were vaccinated by i.m. EP, with each animal receiving 500 μg of the empty-vector plasmid DNA or 500 or 50 μg of VEEVCO DNA at days 0 and 56. Neutralizing antibodies were detected in all of the macaques after a single vaccination with either dose of the VEEVCO vaccine, and a second vaccination significantly boosted the mean PRNT80 titers (Fig. 7). In addition, the mean PRNT80 titers were not significantly different between the 500- and 50-μg dose groups at any of the time points.

On day 112, the macaques were challenged with VEEV IAB strain Trinidad donkey by the aerosol route and the average inhaled dose was calculated to be $5 \times 10^6$ PFU or $300 \text{ median effective doses (ED}_{50}$). Serum viremia was detected in macaques vaccinated with the control empty vector on day 1, peaked on day 2 with a mean group titer of 1,576 PFU/ml, and persisted for 4 days postchallenge (Fig. 8A). In contrast, the macaques that received either dose of the VEEVCO vaccine, and a second vaccination significantly boosted the mean PRNT80 titers (Fig. 7). In addition, the mean PRNT80 titers were not significantly different between the 500- and 50-μg dose groups at any of the time points.

On day 112, the macaques were challenged with VEEV IAB strain Trinidad donkey by the aerosol route and the average inhaled dose was calculated to be $3 \times 10^6$ PFU or $300 \text{ median effective doses (ED}_{50}$). Serum viremia was detected in macaques vaccinated with the control empty vector on day 1, peaked on day 2 with a mean group titer of 1,576 PFU/ml, and persisted for 4 days postchallenge (Fig. 8A). In contrast, the macaques that received either dose of the VEEVCO DNA vaccine were aviremic in all of the postchallenge serum samples tested.

The protective efficacy of the vaccine was also assessed by telemetry monitoring of the postchallenge fever responses. Macaques vaccinated with the empty-vector DNA developed high biphasic fevers that appeared within 24 h of VEEV chal-
and had peaks at days 2 and 6 postchallenge (Fig. 8B). Macaques that received either 500 or 50 μg of the VEEV CO DNA vaccine also developed fevers after a VEEV aerosol challenge, but these peaked at day 3 postchallenge and the mean temperature elevations were significantly lower than those of macaques that received the empty-vector DNA at day 1 (P < 0.05), day 2 (P < 0.0001), and day 6 (P < 0.05). In addition, there was no significant difference in the mean temperature elevations of macaques that received the 500- or 50-μg dose of the VEEV CO DNA vaccine at any time (P > 0.05).

To monitor lymphopenia, peripheral blood lymphocyte (PBL) counts determined for postchallenge blood samples collected from all macaques on days 1 to 7 after VEEV challenge were compared to average baseline counts determined for prechallenge blood samples collected on days −3 to −1. The PBL counts of macaques vaccinated with the empty-vector plasmid DNA dropped an average of 58.6% from the baseline within 24 h of VEEV exposure, and these macaques remained lymphopenic for an average of 6 days postchallenge (Fig. 8C). In contrast, the PBL counts of macaques vaccinated with 500 or 50 μg of the VEEV CO DNA vaccine dropped an average of 18.5% and 28.1% from the baseline, respectively, at 24 h after a challenge, and the duration of this lymphopenia averaged 3 days for both groups. Over the entire 7-day postchallenge observation period, the macaques vaccinated with the empty-vector DNA developed a lymphopenia that was characterized by an average change of −27.5% from the baseline PBL counts, while that of macaques vaccinated with 500 or 50 μg of the VEEV CO DNA vaccine was −6.4% and 1.1%, respectively.

The macaques were also monitored for clinical signs of disease after an aerosol VEEV challenge. Macaques vaccinated with empty-vector DNA displayed mild signs of disease, including depression, anorexia, and slightly reduced responses to stimuli beginning on day 2 postchallenge, which coordinated with the time of peak serum viremia and the initial peak of fever responses. On postchallenge days 4 to 6, at the time of the secondary peak of fever responses, increased signs of disease, including hunched posture with the back turned toward the observer and neurological signs including loss of coordination and occasional tremors, were observed in these macaques. By day 8 postchallenge, and corresponding to a reduction in the severity of the fever responses, only mild signs of disease were evident in these macaques, and on day 9 postchallenge, their behavior returned to normal. In contrast, macaques that received either 500 or 50 μg of the VEEV CO DNA vaccine displayed limited clinical signs of disease with only slight activity observed in some macaques on days 3 and 4 postchallenge.

**DISCUSSION**

The primary objective of the studies described here was to develop a protective DNA vaccine for VEEV. Our ultimate goal is to create a combination DNA vaccine for VEEV, EEEV, and WEEV. For this, we need both a potent vaccine and a flexible, effective delivery method. Our earlier work demonstrated that a DNA vaccine that expressed the entire structural protein coding region (C-E3-E2-6K-E1) of VEEV IAB strain Trinidad donkey administered to mice and nonhuman primates by PMED provided partial protection against an aerosol VEEV challenge. To improve this outcome, we sought to identify a vaccination strategy that encompassed both a highly immunogenic vaccine construct and a robust delivery method. A refined DNA vaccine expressing VEEV envelope glycoprotein genes that were modified to maximize mammalian codon availability and to remove viral elements shown to compromise expression was developed. EP was selected as the means for delivery because it has been shown to effectively elicit immune responses to a number of antigens in a variety of animals, as well as in humans. Importantly for our work, it is compatible with the delivery of higher DNA doses and volumes of injection that will likely be required to achieve our downstream goal of a combination encephalitic alphavirus vaccine.

Using the modified VEEV CO DNA vaccine and i.m. EP delivery, we observed significantly higher ELISA titers at low DNA doses than with our previous DNA vaccine in mice. Of
greater impact, the modified DNA vaccine elicited higher levels of neutralizing antibodies at all of the doses tested and with fewer vaccinations than did the older construct. Although the basis for the improved neutralizing antibody response of the modified vaccine was not exhaustively studied here, there are two likely reasons for this observation. First, previous reports have shown that the capsid protein of VEEV can be cytotoxic and can inhibit cellular transcription and nuclear import in vertebrate cells (2, 16). Consistent with these findings, the results of our immunogenicity and challenge studies favored the DNA vaccine in which capsid was deleted. Second, because the majority of VEEV-neutralizing antibodies are known to be directed against epitopes present in the E2 glycoprotein (22, 43), codon optimization likely resulted in better expression of the E2 gene, due to either improved mRNA stability or better availability of tRNAs for the codons present in mammalian cells. Again, although we have not completed directly investigating these possibilities, we are finding improved in vitro expression of E2 in transiently transfected mammalian cells, as measured by using a monoclonal antibody to E2 and flow cytometry (data not shown). Currently, we are conducting additional studies to attempt to more clearly define properties of VEEVC, that contribute to its robust immunogenicity.

In addition to improvements noted with the DNA vaccine construct, we also found that both the total IgG and neutralizing antibody titers against VEEV were significantly higher in mice that received the vaccine by EP than in those that received it by i.m. injection only. These results indicate that EP clearly enhances the immunogenicity of VEEVC. Moreover, in the studies presented here, we observed complete protection of mice vaccinated by i.m. EP with VEEVC from a lethal aerosol VEEV challenge. As the most widely accepted correlate of protective immunity against VEEV is antibody responses directed against the envelope glycoproteins (4, 19, 35), the increased neutralizing antibody responses that we obtained with VEEVC delivered by i.m. EP is the most likely explanation for the observed improved protection. Nevertheless, there have been reports that serum antibodies do not always correlate with protection from an aerosol VEEV challenge. For example, in one passive-transfer study, mucosal antibodies were found to be required for the protection of mice from an aerosol challenge (14). In this study, we did not attempt to measure antibodies present in the nasal or respiratory tract; however, our results clearly indicate that i.m. EP vaccination can elicit protective immunity in mice.

Although cytotoxic T-lymphocyte activity has not been detected in animals vaccinated against VEEV, certain populations of T lymphocytes have been reported to contribute to protection against VEEV in mice (6, 25, 34, 52). In one study with mice with nonfunctional B cells but normal T-cell activity and cytokine production, complete protection could be achieved only when the passively administered intranasal antibody was accompanied by vaccination with TC-83 (14). The results were interpreted to indicate that the cell-mediated immune responses induced by vaccination with the live-attenuated virus were necessary to achieve protection from aerosol challenges. In our study, we showed a balanced IgG1-IgG2a antibody response to VEEV, which is predictive of a balanced Th1-Th2 immune response. A caveat of these results was the measurement of the antibody subtypes in pooled sera rather than in individual mice, which was necessitated by the limited amount of serum that can be obtained from a mouse. However, our additional results indicate that both the humoral and cellular arms of the immune response were triggered. Support for this comes from our demonstration of IFN-γ responses in ELISPOT assays using restimulated splenocytes from mice that received VEEVC. Furthermore, our observation that the neutralizing antibody titers of rabbits could be significantly boosted approximately 6 months after the initial vaccination indicates that memory immune responses can be generated with the VEEVC DNA vaccine.

To evaluate the protective efficacy of VEEVC, we used an established and well-characterized aerosol challenge model of VEEV infection of cynomolgus macaques which has previously been used to assess the protection of numerous live-attenuated and formalin-inactivated VEEV vaccines (37-40). In a previous study using this model, we found that cynomolgus macaques vaccinated with three doses of wild-type VEEV DNA delivered by PMED developed low levels of VEEV-neutralizing antibodies and were only partially protected from an aerosol VEEV challenge (12). In the present studies, we demonstrated that cynomolgus macaques that received two EP vaccinations with a dose as low as 50 µg of VEEVC DNA developed substantial VEEV-neutralizing antibody titers. In addition, these macaques had no detectable serum viremia and had reduced febrile reactions, lymphopenia, and clinical signs of disease compared to those of negative-control macaques after an aerosol VEEV challenge. For ethical reasons, we do not routinely sacrifice macaques that survive and completely recover after a VEEV challenge. Although this prevents us from completely ruling out the possibility of viral incursion into the brain, the macaques vaccinated with the VEEVC DNA displayed no neurological signs of disease after challenge. To our knowledge, this represents the first report of a DNA vaccine that can provide similar protection against viremia and manifestation of disease symptoms as observed for TC-83 and C-84 in this VEEV aerosol challenge model. Further, it is encouraging that this protection was achieved with a relatively low DNA dose, as this increases the prospect that these results can be extrapolated to humans.

While the improved immunogenicity associated with i.m. EP in these studies is encouraging, the tolerability profile of this administration procedure will be an important element of clinical feasibility. Accumulated clinical experience with the EP device used in the studies presented here indicates that the procedure is associated with more discomfort than a conventional i.m. injection but is not outside the range of acceptability for use in prophylactic immunization (49). Further developments to improve tolerability, including dermal rather than i.m. administration, may further facilitate the use of this promising vaccine approach.

Taken together, the results of our studies indicate that the VEEVC DNA vaccine delivered by i.m. EP is able to elicit robust and durable protective immune responses with low DNA doses and few vaccinations. These findings suggest that this vaccine may represent a suitable alternative to the live-attenuated and formalin-inactivated VEEV vaccines currently used under IND status. Moreover, our results, along with those of others (5, 41, 49), recommend EP delivery as an effective method to elicit immunity in animals and humans, with a
capacity large enough to accommodate multiple vaccines in a single administration.

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