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Naked DNA Vaccines Expressing the prM and E Genes of Russian Spring Summer Encephalitis Virus and Central European Encephalitis Virus Protect Mice from Homologous and Heterologous Challenge

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Naked DNA vaccines expressing the prM and E genes of two tick-borne flaviviruses, Russian spring summer encephalitis (RSSE) virus and Central European encephalitis (CEE) virus were evaluated in mice. The vaccines were administered by particle bombardment of DNA-coated gold beads by *Accell* gene gun inoculation. Two immunizations of 0.5 to 1 μg of RSSE or CEE constructs/dose, delivered at 4-week intervals, elicited cross-reactive antibodies detectable by enzyme-linked immunosorbent assay and high-titer neutralizing antibodies to CEE virus. Cross-challenge experiments demonstrated that either vaccine induced protective immunity to homologous or heterologous RSSE or CEE virus challenge. The absence of antibody titer increases after challenge and the presence of antibodies to E and prM, but not NS1, both before and after challenge suggest that the vaccines prevented productive replication of the challenge virus. One vaccination with 0.5 μg of CEE virus DNA provided protective immunity for at least 2 months, and two vaccinations protected mice from challenge with CEE virus for at least 6 months.

Tick-borne encephalitis (TBE) occurs over a wide area of Europe and the former Soviet Union. TBE is most frequently caused by infection with the flaviviruses Central European encephalitis (CEE) virus and Russian spring summer encephalitis (RSSE) virus. These viruses are antigenically and genetically closely related to one another and often are considered to be subtypes of the same virus. However, two different tick vectors transmit RSSE and CEE viruses (*Ixodes persulcatus* and *Ixodes ricinus*, respectively), and RSSE virus generally causes a more severe disease than does CEE virus (reviewed in reference 33). Also, RSSE and CEE viruses can be distinguished by cross-neutralization (5) and by other serological tests (reviewed in reference 4).

In parts of Europe, TBE cases have notably declined since the introduction in 1976 of a formalin-inactivated, chicken embryo-derived vaccine. The vaccine is based on an Austrian strain of CEE virus and elicited protective immunity in mice to the homologous CEE virus (strain Hypr) and to four strains of RSSE virus (18). Despite the success of this vaccine, it suffers the disadvantages commonly associated with inactivated virus vaccines such as the requirement for large-scale production and purification of a highly infectious human pathogen, the risk of incomplete inactivation of the virus, and the need to deliver the vaccine with adjuvant in a three-shot series (26). Also, this vaccine is not licensed for use in the United States.

For these reasons, we are interested in developing an improved TBE vaccine. In this report, we describe two plasmid-based TBE candidate vaccines which express the premembrane (prM) and envelope (E) genes of RSSE or CEE virus under control of a cytomegalovirus early promoter. We chose the prM and E genes for expression because of earlier reports with other flaviviruses which indicated that coexpressed prM and E form subviral particles that are able to elicit neutralizing and protective immune responses in animals (22, 24, 35). Coexpression of prM and E of CEE virus also produced subviral particles that retained biological properties of complete virus such as membrane fusion and hemagglutination (39) and which were immunogenic in mice (15).

To deliver our DNA vaccines, we chose to use the *Accell* gene gun (patent application W0 95/197991) (Geniva, Madison, Wis.). This instrument, which delivers DNA-coated gold beads directly into epidermal cells by high-velocity particle bombardment, was shown to more efficiently induce both humoral and cell-mediated immune responses, with smaller quantities of DNA, than inoculation of the same DNAs by other parenteral routes (8, 11, 13, 34). Epidermal inoculation of the DNA candidate vaccines also offers the advantages of gene expression in an immunologically active tissue that is generally exfoliated within 15 to 30 days and which is an important natural focus of viral replication after tick bite (2, 27, 36, 40). The experiments described here were intended to evaluate the elicitation of cross-protective immunity to RSSE and CEE viruses by DNA vaccines.

MATERIALS AND METHODS

Viruses, cells, and medium. Viruses were kindly provided by Robert Shope, Yale Arbovirus Research Unit, New Haven, Conn. Cell lines were obtained from the American Type Culture Collection. CEE virus, strain Hypr, was originally isolated in 1953 from a TBE patient in Czechoslovakia. RSSE virus, strain Sofjin, was originally isolated in 1937 from a TBE patient from the far eastern USSR (4). Langat virus was originally isolated in 1956 from ticks collected in Malaysia. RSSE and CEE viruses were propagated in Vero E6 cells, and Langat virus was propagated in LLC-MK_2 cells. Cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics. Propagation and assay of RSSE or CEE virus were carried out in a biosafety level 4 laboratory.

Cloning of the prM and E genes of RSSE and CEE viruses. For reverse transcription (RT)-PCR amplification of the prM and E genes of RSSE and CEE viruses, specific oligonucleotide primers were designed to correspond to se-

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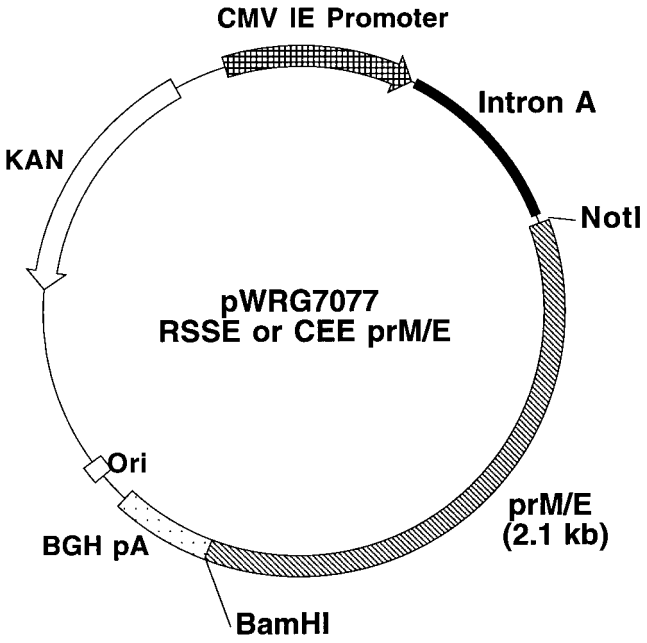

FIG. 1. Schematic of pWRG7077 containing prM and E genes of RSSE and CEE viruses. Genes were amplified by RT-PCR and cloned into *Not*I and *Bam*HI sites of pWRG7077. Characteristics of pWRG7077 are similar to those of pWRG1602 described previously (8) and include a human cytomegalovirus immediate-early (CMV IE) promoter, intron A, a bovine growth hormone transcription terminator and polyadenylation signal (BGH pA), and a kanamycin resistance gene.

quences previously reported for RSSE and CEE viruses (Genbank U39292 and X03870, respectively). For the forward primers, nucleotides were modified around the translation initiation codons (bold type below) to generate sequences with a favorable context for translation initiation (25). The forward and reverse primers for RSSE virus were 5′GCAGTAGAC**A**GGA**TG**GGTTGGTTG3′ and 5′GCACAGCCAACTTAAGCTCCCACTCC3′, respectively. The forward and reverse primers for CEE virus were 5′GCGACGGAC**A**GGATG**G**GCTGGTT GCTAG3′ and 5′CACAGCGCAGCCAACTTACGCCCCCACTCC3′, respectively.

Total intracellular RNA of virus-infected Vero cells was extracted by using Trizol reagent (Gibco). For RT of the RSSE and CEE virus prM and E genes, the specific oligonucleotide primers and/or random primers were used with Superscript cDNA synthesis reagents (Gibco). The same specific primers were used to amplify the cDNA by PCR with Expand HiFi reagents (Boehringer Mannheim). PCR was carried out in a PCR 9600 thermocycler (Perkin-Elmer). PCR conditions were 40 cycles of 94°C for 40 s, 38°C for 45 s, and 72°C for 1 min, after which reactions were incubated at 72°C for 5 min and then held at 4°C until used for cloning into the pCRII plasmid (Invitrogen). After verification of orientation, the cDNA inserts were excised from pCRII by digestion with *Eco*RV and *Spe*I or by digestion with *Not*I and partial digestion with *Bam*HI. The RSSE and CEE virus cDNAs were then cloned into the *Hin*dIII (blunt) and *Nhe*I sites of pJW4303 (28) or the *Not*I and *Bam*HI sites of pWRG7077 (Fig. 1).

Transient-expression assays of RSSE and CEE virus prM and E genes. For each assay, 5 μg of pWRG7077 containing RSSE or CEE virus prM and E genes or control plasmid with no insert was mixed with 200 μl of OptiMEM medium (Gibco) with no antibiotics. A separate solution was prepared consisting of 40 μl of Lipofectin reagent (Gibco) in 200 μl of OptiMEM (Gibco). Both solutions were incubated at room temperature for 30 to 45 min, after which they were combined and incubation was continued at room temperature for 10 to 15 min. OptiMEM (1.6 ml) was then added to each assay, and the solution was placed onto monolayers of COS cells in 25-cm^2 flasks that had been rinsed one time with 2 ml of serum-free EMEM. The cells were incubated for 7 h at 37°C, and then the Lipofection-DNA solution was removed and fresh OptiMEM with antibiotics was added and incubation was continued. At 26-h postinfection, the medium was removed from the cell cultures and replaced with EMEM without cysteine or methionine. After incubation for 1 h at 37°C, 200 μCi of ^{35}S-labeled Promix (methionine and cysteine; Amersham) was added to each flask and the cells were incubated for 4 h at 37°C. The radiolabeling medium was then removed, and cells were lysed on ice with 1 ml of a buffer consisting of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.5 M NaCl, 4% Zwittergent 3-14 (Calbiochem-Behring), and protease inhibitors (Boehringer Mannheim). Cell nuclei were removed by centrifugation for 5 min at 12,000 × *g* in a microcentrifuge. An aliquot (100 μl) of each supernatant was mixed with 5 μl of a hyperimmune mouse ascitic fluid to RSSE or CEE virus. After incubation on ice overnight, 100 μl of 50% protein A-Sepharose (Sigma) in lysis buffer was added to each tube, and the samples were shaken at 4°C for 30 min. The Sepharose beads were recovered by centrifugation in a microcentrifuge and were washed three times with lysis buffer and one time with 10 mM Tris-HCl, pH 8.0. The beads were then boiled for 2 min in protein sample buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) as described previously (1).

Preparation of gene gun cartridges, immunization, and challenge of mice. Plasmid DNA was precipitated onto the outside surface of gold beads (approximately 2 μM in diameter) as described previously (8). The DNA loads were 0.5 to 1 μg/mg of gold. The DNA-coated gold particles were dried on the inside walls of Tefzel tubing, which was then cut into 0.5-in sections to make cartridges for the gene gun (34). These cartridges each contained approximately 0.5 mg of gold coated with 0.25 to 0.5 μg of DNA. BALB/c mice (approximately 6 to 8 weeks old) were immunized by using the hand-held, helium-powered *Accell* gene gun to deliver approximately 0.5 to 1 μg of DNA to the epidermis as described in Results and as reported previously (34). For challenge studies, mice were transferred to a biosafety level 4 containment area and challenged by intraperitoneal inoculation of approximately 50 PFU of suckling mouse brain-passaged RSSE or CEE virus, a dose previously determined to be approximately 100 times the 50% lethal dose (LD_{50}) for BALB/c mice. Mice were observed daily for signs of illness and for death. This research was conducted in accordance with procedures described in the *Guide for the Care and Use of Laboratory Animals* (prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources Commission of Life Sciences-National Research Council) (33a). The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

ELISA. Direct immunoglobulin G enzyme-linked immunosorbent assay (ELISA) was performed by using methods similar to those described previously (6, 32). The viral antigen was prepared by detergent lysis of RSSE or CEE virus-infected Vero cells, and infectious virus was inactivated by gamma irradiation of lysates (6). One half of a 96-well polyvinylchloride microtiter plate (Dynatech, Vienna, Va.) was coated directly with 100 μl of viral antigen/well diluted in 0.01 M phosphate-buffered saline (pH 7.4) with 0.01% thimerosal (coating buffer) at a predetermined optimal dilution (1:1,000). The other half was coated with 100 μl/well of a similarly treated negative antigen made from uninfected cells. Plates were wrapped in plastic wrap and incubated at 4°C overnight. The next day plates were washed three times with wash buffer (coating buffer and 1% Tween-20; 300 μl/well/wash) by using an automatic plate washer (Biotek Instruments). All subsequent reagents added to the plates were diluted in wash buffer containing 5% skim milk (Difco). After the addition of each reagent, the plates were incubated in a moist environment at 37°C for 1 h and then washed three times. Serum samples were initially diluted in microtiter tubes (Bio-Rad) and then further diluted from the microtiter tube into wells coated with either positive or negative antigen (final dilution, 1:100). Sera were screened at a 1:100 dilution or were serially diluted fourfold from 1:100 to 1:6,400 in the ELISA plate. The positive-control sera used were ascitic fluids from hyperimmunized mice inoculated with authentic homologous virus. Negative-control sera used were prebleeds and controls from mice used in the study. After incubation, plates were washed and 100 μl of horseradish peroxidase-labeled goat anti-mouse immunoglobulin G antibody (200 ng/ml; Boehringer Mannheim) was added to each well. The substrate 2,2′-azino-di-3-ethylbenthiazoline sulfonate (ABTS; Kirkegaard and Perry) was added, and plates were read at 410 nm with a Dynatech MR5000 reader and Lotus Measure. The readings were adjusted by subtracting the optical density (OD) of the negative antigen-coated wells from that of the positive antigen-coated wells. OD cutoff values were determined as follows. The mean of the adjusted OD values was determined for all the mouse prebleed and control samples and the standard deviation was calculated. The cutoff of the assay was the mean OD value plus 3 standard deviations rounded up to the nearest tenth. An OD value was considered positive if it was greater than or equal to this value. The titer was equal to the reciprocal of the last dilution that was above or equal to the OD cutoff value. A serum sample was considered positive if the titer was ≥ 1:100.

PRNT assays. Twofold dilutions of sera (1:20 to 1:640) were prepared in EMEM supplemented with 10% FBS and antibiotics. Dilutions were incubated at 56°C for 30 min to inactivate complement and then were mixed with an equal volume of infectious RSSE or CEE virus in EMEM supplemented with 10% FBS and antibiotics to yield a mixture containing approximately 500 PFU of virus/ml. The virus-antibody mixtures were incubated at 37°C for 1 h and then stored at 4°C overnight. The following day, 0.2 ml of the mixture was added to duplicate wells of six-well plates containing confluent monolayers of Vero E6 cells. The plates were incubated for 1 h (rocking gently every 15 to 20 min). The wells were then overlaid with 2 ml of 0.6% Seakem ME agarose (FMC Corp.) prepared in EMEM and supplemented with 5% FBS, nonessential amino acids, L-glutamine, and antibiotics. The plates were incubated at 37°C in 5% CO_2 for 6 days, after which a second overlay of 0.5% agarose in EMEM supplemented with 2.5% FBS and neutral red was applied. Plaques were visible 1 to 2 days later. The neutralizing antibody titer was calculated as the reciprocal of the highest dilution resulting in an 80% reduction of plaques (80% plaque reduction neutralization titer [$PRNT_{80}$]) compared to a control of virus with no added antibody.

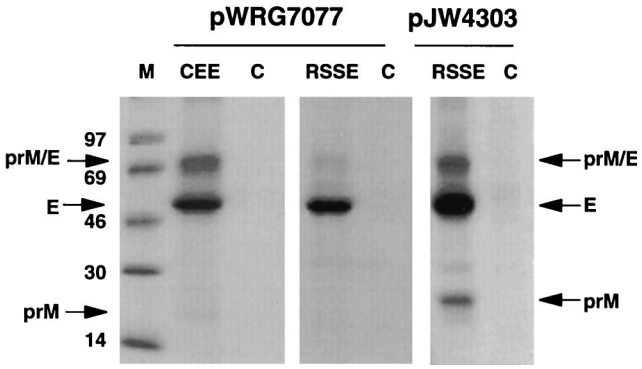

FIG. 2. Transient expression of naked DNA plasmids in COS cells. Plasmids containing the RSSE or CEE virus prM and E genes or plasmids with no inserted gene (C) were transfected into COS cells and expression products were immune precipitated with antibodies to RSSE or CEE virus. Products were analyzed by PAGE and autoradiography. The positions of E, prM, and uncleaved prM and E (prM/E) are indicated. The sizes (kDa of molecular mass markers (M) are shown on the left.

Radiolabeling and immune precipitation of Langat virus proteins. Conditions for infection and radiolabeling of Langat virus proteins with [^{35}S]methionine were described previously (19). Briefly, Langat virus-infected LLC-MK$_2$ cell monolayers in 25-cm^2 flasks were radiolabeled 18 to 24 h after infection with 200 μCi of ^{35}S-labeled ProMix/ml. The cells were lysed in a buffer consisting of 400 mM NaCl, 50 mM Tris HCl (pH 8.0), 1 mM EDTA, 1% Triton X-100, 0.2% deoxycholate, and protease inhibitors. Cell nuclei were removed by centrifugation. Langat virus proteins were immune precipitated with 2 to 5 μl of experimental mouse sera and analyzed by sodium dodecyl sulfate-PAGE.

RESULTS

Cloning and transient expression of prM and E genes. Expression of the prM and E genes of RSSE and CEE viruses was assayed by transfection of plasmid pJW4303 (28) or pWRG7077 (Fig. 1), containing the RSSE genes, or pWRG7077, containing the CEE genes, into cell cultures. Each of the constructs produced E, prM, and uncleaved prM/E which could be immune precipitated with antibodies to authentic viral proteins (Fig. 2). The cleavage of prM and E is thought to occur by cellular signal peptidase and that of prM and M by the action of another host enzyme, perhaps furin (37).

Antigenicity of the candidate vaccines. BALB/c mice were immunized by delivery of DNA-coated gold beads to the abdominal epidermis by particle bombardment with helium pressure by using the *Accell* gene gun. For our first experiment and the first immunization of the second experiment, we used RSSE virus prM-E cloned into pJW4303 (28). For all subsequent studies we used RSSE or CEE virus prM-E cloned into pWRG7077 (Fig. 1). The two plasmids have the same control elements, i.e., a human cytomegalovirus early promoter, intron A, and a bovine growth hormone polyadenylation-transcription termination signal. However, pWRG7077 does not contain the simian virus 40 origin of replication and it has a kanamycin resistance gene rather than an ampicillin resistance gene and is therefore more suitable for the development of human vaccines.

In our initial experiment, 10 mice were immunized with the RSSE virus construct and 5 mice were immunized with pJW4303 with no insert. Each mouse received two shots in adjacent sites with a combined total of approximately 1 μg of DNA. Four weeks after the first immunization, the mice were bled and a second immunization of two shots was given. Four weeks after the second immunization, the mice were bled again and sera were assayed by ELISA. All of the mice vaccinated with the RSSE virus construct had detectable responses to RSSE virus after one vaccination and all of them had increased responses after the second vaccination (Fig. 3A). None of the serum samples from the control mice displayed any reactivity with RSSE virus antigen (Fig. 3A).

To assess the ability of the RSSE virus DNA to elicit an antibody response to CEE virus, we performed a second experiment in which 10 mice were immunized as before with RSSE virus DNA and 5 mice were immunized with plasmid with no insert. Four weeks after the second vaccination, an ELISA was performed with RSSE or CEE virus antigen. Antibody responses were detected to both antigens with sera from all vaccinated mice (Fig. 3B).

To further evaluate the ability of the RSSE and CEE virus DNAs to elicit cross-reactive antibody responses, we performed a third experiment, in which we immunized 20 mice with RSSE virus DNA, 16 mice with CEE virus DNA, 16 mice with both RSSE and CEE virus DNA, and 18 mice with plasmid with no insert. As before, two immunizations (each consisting of two gene gun shots) were given at 4-week intervals, but the DNA dose was reduced from 1 μg to 0.5 μg at each immunization. The mice were bled 4 weeks after the second immunization, and serum samples were assayed by ELISA. Unexpectedly, we found that although there was an initial response to the antigen, there was not a rise in response after the second immunization (not shown). From other experiments, we knew that 0.5 μg of these DNAs was sufficient to elicit antibody responses in mice (not shown). Based on these results and those of other studies (not shown) we determined that a hardware modification to the gene gun (a brass insert which altered the helium flow and was intended to more evenly disperse the gold beads at the target inoculation site) resulted in reduced antigenicity. Consequently, we immunized the mice once more (4 weeks after the second immunization) with the RSSE, CEE and RSSE, or CEE virus DNAs. The mice were then bled, and ELISA titers of sera were determined for both RSSE and CEE virus antigen-coated plates (Fig. 4). As in earlier experiments, increases in ELISA titers were observed in the majority of the samples after this final immunization. The CEE virus antigen used to coat the ELISA plates was apparently not as concentrated as the RSSE virus antigen in that

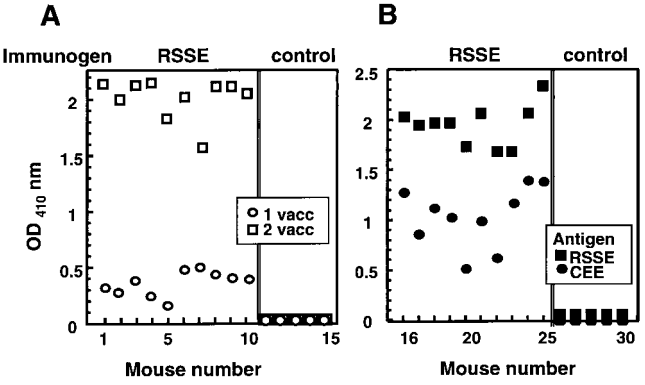

FIG. 3. Antibody responses of mice to naked DNA vaccines as detected by ELISA. (A) Mice were immunized two times, 4 weeks apart, with 1 μg of pJW4303/dose expressing the prM and E genes of RSSE virus. ELISA of RSSE virus antigen-coated plates containing sera collected just before the second immunization (1 vacc) or 4 weeks after the second immunization (2 vacc) was performed. (B) Mice were immunized once with 1 μg of pJW4303 expressing the RSSE virus prM and E genes, and 4 weeks later, were immunized once with 1 μg of pWRG7077 expressing the RSSE virus prM and E genes. ELISA of RSSE or CEE virus antigen-coated plates containing sera collected 4 weeks after the second immunization was performed. Controls for each experiment were comparable plasmids with no gene insert.

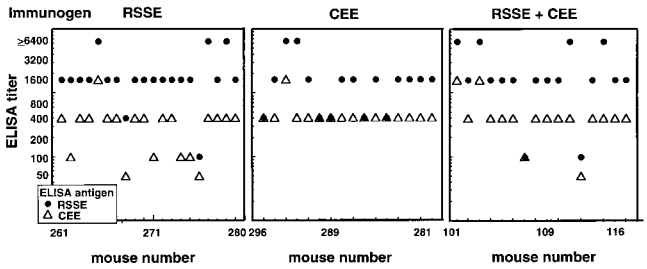

FIG. 4. ELISA titers to RSSE and CEE viruses of mice immunized with RSSE, CEE, or RSSE and CEE virus DNAs. Mice were immunized three times at 4-week intervals with 0.5 μg of DNA/dose. Titers of sera were determined 4 weeks after the final immunization.

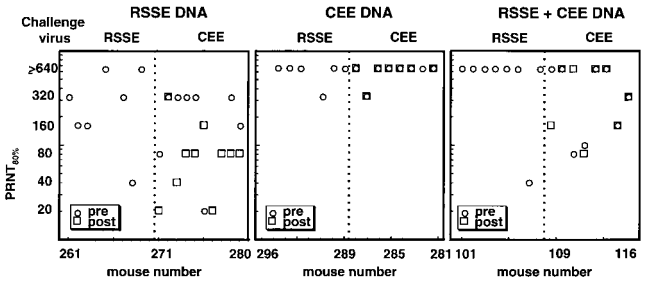

FIG. 5. Plaque reduction neutralization by pre- and postchallenge sera of mice immunized with naked DNA vaccines expressing the prM and E genes of RSSE, CEE, or RSSE and CEE viruses. Twofold dilutions of sera from 1:20 to 1:640 were used in PRNT assays with CEE virus. PRNTs are listed as the greatest dilution of serum which resulted in ≥80% reduction of the number of plaques observed in controls incubated with serum from mice vaccinated with control plasmids.

titers were uniformly lower with sera from both RSSE and CEE virus DNA-immunized mice (Fig. 4).

Protective efficacy of the candidate vaccines. To determine if the DNA vaccines could protect mice from challenge with virulent RSSE and CEE viruses, we challenged mice from various experiments with either virulent RSSE or virulent CEE virus. A summary of the challenge results from four experiments is shown in Table 1. All 55 of the mice immunized with plasmids containing the RSSE or CEE virus genes remained healthy after virus challenge. In contrast, all 27 control mice (18 immunized with plasmid lacking an insert and 9 nonimmunized mice) displayed symptoms of infection after virus challenge; 14 of 17 mice died after challenge with RSSE virus, and 8 of 10 mice died after challenge with CEE virus.

Neutralizing antibody and sterile immunity. Neutralizing antibodies correlate with protective immunity to tick-borne flaviviruses, as has been demonstrated in mice by passive transfer of neutralizing monoclonal antibodies to M and E (16, 19). We measured the neutralizing antibody responses elicited by the vaccines in mice from the third experiment just before challenge. Because we found that CEE virus produced clearer, more easily discernible plaques than did RSSE virus, and because infectious virus assays required biosafety level 4 containment, we performed all $PRNT_{80}$ assays only with CEE virus. $PRNT_{80}$s to CEE virus were higher for mice immunized with CEE virus DNA or with RSSE and CEE virus DNA than for those immunized only with RSSE virus DNA. Such results are consistent with previous studies which differentiated RSSE virus and CEE virus on the basis of cross-neutralization with polyclonal sera (5). We found that all of the mice except one had prechallenge neutralizing antibody titers of ≥40 (Fig. 5). For samples in which an endpoint titer was reached, postchallenge neutralizing antibody titers were generally the same as or lower than prechallenge titers, suggesting a protection from infection (Fig. 5). For samples with prechallenge titers of ≥640, we also assayed pooled sera to estimate an endpoint titer. For the pooled serum samples from the CEE virus challenge group (mice 109 to 116), the $PRNT_{80}$s were 1,280 both before and after challenge. These results are also consistent with abortive infection by the challenge virus. The same results were obtained with postchallenge sera from the RSSE virus challenge group, i.e., the same or lower titers after challenge, but the results are not included in Fig. 5 because of the problem mentioned above with regard to invalid controls.

As another means to measure sterile immunity, we immune precipitated radiolabeled Langat virus proteins with sera from mice vaccinated with RSSE or CEE virus DNA both before and after challenge with RSSE or CEE virus. Langat virus was used rather than RSSE or CEE virus for these experiments because we previously demonstrated that Langat proteins are cross-reactive with RSSE and CEE virus antibodies (19) and because we were able to use Langat virus at biosafety level 3 containment rather than at level 4. We expected that vaccinated mice would have antibodies to Langat virus E but not NS1. Postchallenge sera would also display reactivity only with E if the mice were not productively infected with the challenge viruses. If they were infected, however, we expected to also see reactivity with NS1. In addition to pooled samples, individual sera from mice 107 and 112 were assayed as representatives of samples for which a higher postchallenge than prechallenge PRNT was observed. Analysis of the immune precipitation products by PAGE revealed that although both E and NS1 proteins were clearly precipitated by polyclonal hyperimmune mouse ascitic fluids to RSSE or CEE virus, NS1 was not evident in any of the pre- or postchallenge sera from experimental mice (Fig. 6). The pooled sera from mice 109 to 116 did have a faint band in the area expected for NS1 (Fig. 6A), so these

TABLE 1. Mortality of mice immunized with RSSE, CEE, or RSSE and CEE virus naked DNA vaccines and challenged with RSSE or CEE virus

Virus(es) used for vaccine	Challenge virus	No. dead/total no.						P^a
		Replicate 1		Replicate 2		Overall		
		Vaccinated	Control	Vaccinated	Control	Vaccinated	Control	
CEE	CEE	0/7	6/8			0/7	6/8	0.006
CEE	RSSE	0/10	9/9			0/10	9/9	0.00001
RSSE	CEE	0/5	2/2	0/10	6/8	0/15	8/10	0.0006
RSSE	RSSE	0/10	2/5	0/10	3/3	0/20	5/8	0.0003
RSSE + CEE	CEE	0/8	6/8			0/8	6/8	0.002

[a] Values determined with the test for homogeneity of odds ratios by using the StatXact-Turbo program from Cytel Software Corp., Cambridge, Mass.

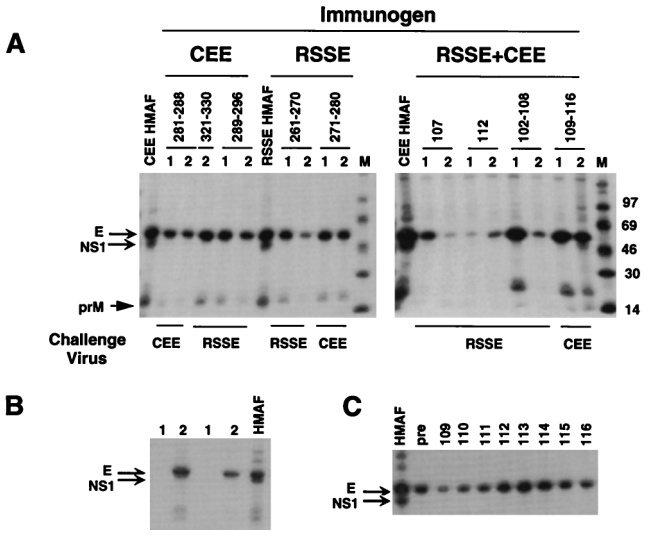

FIG. 6. Immune precipitation of radiolabeled Langat virus proteins with pre- (lanes 1) and postchallenge (lanes 2) sera from mice vaccinated with naked DNA vaccines expressing the prM and E genes of CEE, RSSE, or RSSE and CEE viruses. Immune precipitation products were analyzed by PAGE and autoradiography. Control sera were hyperimmune mouse ascitic fluids (HMAF) to authentic RSSE or CEE virus. The mouse numbers shown above each autoradiograph correspond to those in Fig. 4 and 5. (A) Immune precipitation results obtained with pooled sera, except for those labeled 107 and 112, which are individually analyzed serum samples. (B) Immune precipitation results for sera from the two controls that survived challenge with CEE virus. (C) Immune precipitation results from individual serum samples in group 109 to 116. The sizes (kDa) of molecular mass markers (M) are indicated on the right of panel A.

samples were assayed individually for reactivity with Langat virus proteins. None of these samples immune precipitated Langat virus NS1 (Fig. 6C). Therefore, the immune precipitation results and the PRNT results both suggest that sterile immunity was induced in the vaccinated mice.

Duration of protective immunity after vaccination. We examined the length of immunity induced by one or two vaccinations with approximately 0.5 μg of DNA given at various intervals as described in Table 2. Individual serum samples were analyzed by ELISA, and geometric mean titers of each group were calculated for samples collected immediately before a subsequent vaccination or immediately before challenge (Table 2). All mice that received one vaccination or two vaccinations 4 weeks apart were protected from challenge at 8 weeks after the first vaccination. Two vaccinations, given at 4-, 8-, or 12-week intervals protected all but two mice from challenge 6 months after the initial vaccination (Table 2).

DISCUSSION

The use of nucleic acid vaccines to elicit protective immunity to a variety of viruses has been demonstrated in numerous experimental models (for reviews see references 41–44). In the studies reported here, gene gun administration of microgram quantities of DNA encoding the prM and E genes of RSSE or CEE virus was effective for inducing homologous and heterologous protective immunity in mice. We designed our candidate vaccines to take advantage of earlier findings that showed that coexpressing prM and E results in the formation of secreted antigenic and immunogenic subviral particles (15, 22–24, 35). Such subviral particles, consisting of heterodimers of prM and E, are also a byproduct of normal flavivirus morphogenesis, i.e., the so-called slowly sedimenting hemagglutinins (14, 30, 38). The enhanced immunogenicity of these particles is in part due to the inability of E to assume a native conformation in the absence of prM (22). So, although passively transferred neutralizing monoclonal antibodies to E can protect animals from subsequent flavivirus challenge (3, 12, 16, 19–21, 29, 31), active immunization with expressed, soluble E is not as efficient as prM and E together for inducing protective immunity (15).

As indicated above, neutralizing antibodies to E are, by themselves, sufficient to protect mice, and presumably humans, from CEE virus. Thus, although DNA vaccines delivered to the epidermis by gene gun inoculation efficiently induce both cell-mediated and humoral immune responses (13, 34), we were most interested in analyzing the induction of neutralizing antibodies as a correlate of protection. Our vaccination strategy of two immunizations of 0.5 to 1 μg of DNA delivered at 4-week intervals was based on optimal parameters determined for gene gun inoculation of a reporter gene (8). In those studies, it was determined that microgram quantities of DNA were sufficient for maximal protein expression and the elicitation of antibodies to the expression product. Increasing the amount of DNA from 0.1 to 5 μg of DNA per mg of gold did not result in higher expression levels, and it was suggested that the 300 copies of DNA found on a typical gold bead (0.1 μg DNA/mg of gold) are all that a single cell can efficiently express (8). Although we did not test lesser amounts of DNA, we did investigate other immunization schedules. Our finding that one vaccination with 0.5 μg of DNA can protect mice for at least 2 months and two vaccinations can protect for at least 6 months suggests that the immune response generated is long-lived and offers encouragement for further development of this vaccine for human use.

Interestingly, although all of our challenge studies were performed with 100 LD_{50} of RSSE or CEE virus, some of our control mice that were vaccinated with the plasmid with no

TABLE 2. Antibody responses and duration of protective immunity elicited by CEE virus DNA vaccine[a]

Group	No. of vaccinations (interval [wk])	Wk of challenge	Antibody GMT (range)[b]			No. survived/ no. in group[f]
			1st vaccination[c]	2nd vaccination[d]	Postchallenge	
CEE1	1	8	100 (<100–400)		4,032 (1,600–12,800)	9/9
CEE2	2 (4)	8	<100 (<100)	606 (400–1,600)	1,600 (400–6,400)	10/10
CEE3	2 (4)	24	53.6 (<100–100)	162.5 (<100–400)	2,743 (400–12,800)	9/10
CEE4	2 (8)	24	75.8 (<100–100)	348.2 (100–1,600)	1,600 (400–6,400)	10/10
CEE5	2 (12)	24	ND[e]	75.8 (<100–100)	800 (100–1,600)	4/5

[a] Vaccine administered with gene gun. See Materials and Methods for details.
[b] GMT, geometric mean titer.
[c] Blood samples for groups CEE1 to -4 were collected 8, 4, 4, and 8 weeks after vaccination, respectively.
[d] Blood samples for groups CEE2 to -5 were collected 4, 20, 16, and 12 weeks after the second vaccination, respectively.
[e] ND, not determined.
[f] 0/19 unvaccinated controls survived challenge.

insert did not die (although they did display symptoms of infection). In contrast, all of our unvaccinated control mice died. Although we did not investigate this further, it is possible that enough nonspecific immunity is induced by gene gun inoculation to shift the LD_{50} curve.

The RSSE and CEE virus cross-reactive immunity that we observed was not surprising in that the prM and E polyprotein expression products of the two viruses are 94% identical. Nevertheless, it is known that certain E-specific monoclonal antibodies differentiate RSSE and CEE viruses and that minor changes in E can result in altered neuroinvasiveness in mice (17, 18). Consequently, although either of our DNA vaccines by itself may be sufficient for immunity to TBE-causing flaviviruses, it may be prudent to include both DNAs in a vaccine developed for humans.

In some of our experiments, not only did our candidate vaccines protect mice from death and illness after challenge, but they apparently prevented replication of the challenge virus, as indirectly measured by the absence of antibody titer increases and the absence of NS1-specific antibodies after challenge. Of course, neither of these methods is sensitive enough to detect low levels of virus replication, so it is possible that the challenge virus did establish an infection but was quickly eliminated. If sterile immunity did occur, we assume that it was related to neutralization of the challenge virus by circulating antibodies. Among the mechanistic possibilities for this are prevention of adsorption of virus to host cell receptors or alteration of the conformation of the viral envelope proteins to perturb entry of the virus into the host cell (7). Whichever mechanism occurred, sterile immunity was apparently not required for protective immunity. This is evidenced by the large increases in antibody titers after challenge of some of the mice in our duration of immunity experiments.

In conclusion, we feel that the DNA-gene gun technology offers great promise for a new generation of vaccines for TBE. For the future use of our vaccines, it is imperative that we demonstrate that they can elicit neutralizing and protective responses in primates as well as in mice. The technology is still new and is undergoing constant modifications and revisions. Nevertheless, gene gun inoculation of other DNAs, in quantities similar to those in our studies, effectively induced immune responses in larger animals such as pigs and nonhuman primates (9, 10). Thus, we expect that the amount of DNA needed for successful vaccination will not present a technical barrier. Also, because gene gun delivery of a candidate virus vaccine for hepatitis B (Geniva) was recently approved for use in a human clinical trial, we anticipate no regulatory obstacles for its eventual use in TBE vaccines for humans.

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