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Evaluation of Tick-Borne Encephalitis DNA Vaccines in Monkeys

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Tick-borne encephalitis is usually caused by infection with one of two flaviviruses: Russian spring summer encephalitis virus (RSSEV) or Central European encephalitis virus (CEEV). We previously demonstrated that gene gun inoculation of mice with naked DNA vaccines expressing the *prM* and *E* genes of these viruses resulted in long-lived homologous and heterologous protective immunity (Schmaljohn *et al.*, 1997). To further evaluate these vaccines, we inoculated rhesus macaques by gene gun with the RSSEV or CEEV vaccines or with both DNA vaccines and compared resulting antibody titers with those obtained by vaccination with a commercial, formalin-inactivated vaccine administered at the human dose. Vaccinations were given at days 0, 30, and 70. All of the vaccines elicited antibodies detected by ELISA and by plaque-reduction neutralization tests. The neutralizing antibody responses persisted for at least 15 weeks after the final vaccination. Because monkeys are not uniformly susceptible to tick-borne encephalitis, the protective properties of the vaccines were assessed by passive transfer of monkey sera to mice and subsequent challenge of the mice with RSSEV or CEEV. One hour after transfer, mice that received 50 μl of sera from monkeys vaccinated with both DNA vaccines had circulating neutralizing antibody levels <20–80. All of these mice were protected from challenge with RSSEV or CEEV. Mice that received 10 μl of sera from monkeys vaccinated with the individual DNA vaccines, both DNA vaccines, or a commercial vaccine were partially to completely protected from RSSEV or CEEV challenge. These data suggest that DNA vaccines may offer protective immunity to primates similar to that obtained with a commercial inactivated-virus vaccine.

INTRODUCTION

Tick-borne encephalitis (TBE) is one of the most clinically important arthropod-borne viral diseases in Europe and in the former Soviet Union. TBE occurs in all countries of Central and Eastern Europe, as well as in Scandinavia, France, Italy, Greece, and Albania (reviewed in Monath and Heinz, 1996). Two tick-borne flaviviruses, Russian spring summer encephalitis virus (RSSEV) and Central European encephalitis virus (CEEV), are the primary causes of TBE. The distribution of the viruses is restricted by the range of their tick vectors: *Ixodes persulcatus* for RSSEV and *Ixodes ricinus* for CEEV. Although RSSEV and CEEV are antigenically and genetically similar, with 96% amino acid sequence homology in the envelope (E) proteins, RSSEV causes a more severe disease than does CEEV. Infection with RSSEV has a case fatality rate of approximately 20%, and 30–60% of survivors show neurological sequelae, including paralysis of the shoulder girdle and arms. In contrast, TBE caused by CEEV has a case fatality rate of 1–2%, and serious neurological sequelae are rare (reviewed in Monath and Heinz, 1996).

The use of commercially available vaccines has notably reduced the incidence of TBE in Europe (Kunz *et al.,* 1980). Two vaccines, manufactured in Austria and Germany, are currently available in Europe. Both are chick embryo cell culture-derived CEEV preparations that are inactivated with formalin and are delivered as a three-dose series with adjuvant (Kunz *et al.,* 1980; Heinz *et al.,* 1980; Bock *et al.,* 1990). Despite the success of these vaccines, they have the disadvantages commonly associated with inactivated-virus vaccines such as the requirement for large-scale production of a highly infectious human pathogen, purification, the risk of incomplete inactivation of the virus, and the need to deliver multiple doses of the vaccines in adjuvant. Neither vaccine is licensed for use in the United States, so they are not available for U.S. travelers to TBE endemic regions.

For these reasons, we are interested in developing an improved TBE vaccine. We previously reported the evaluation of naked DNA vaccines for TBE in mice (Schmaljohn *et al.,* 1997). We demonstrated that homologous and heterologous protective immunity could be achieved by gene gun inoculation of plasmid DNA expressing the *prM* and *E* genes of either RSSEV or CEEV. In this study, we evaluated the vaccines in rhesus macaques and compared the results with those obtained with the Austrian commercial inactivated-virus vaccine.

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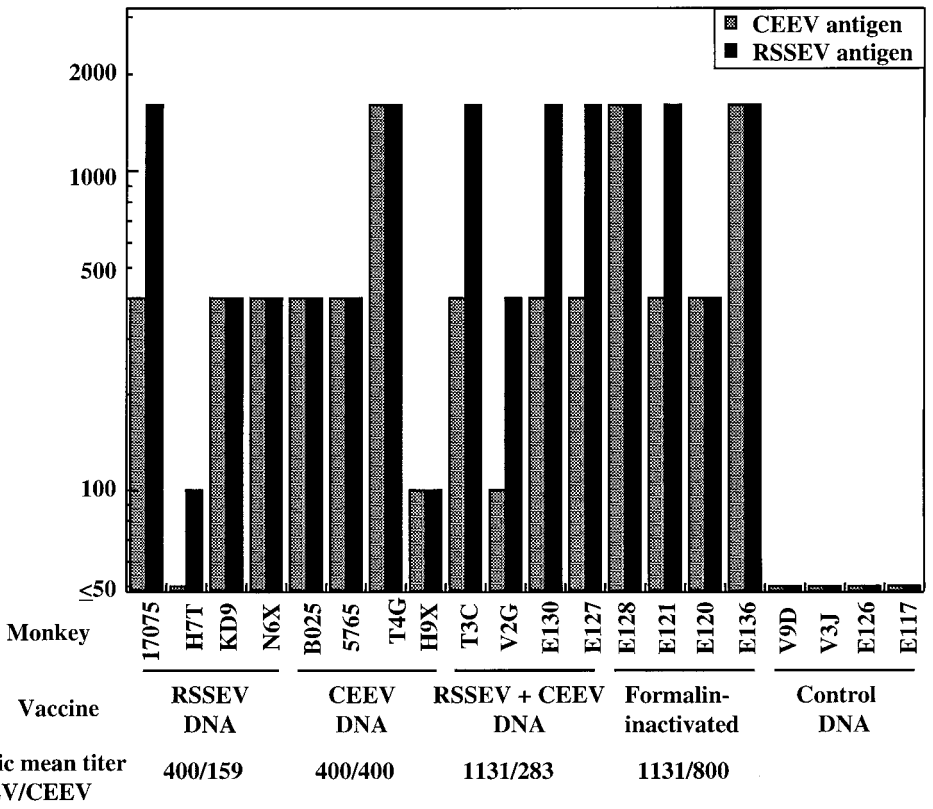

FIG. 1. Direct IgG ELISAs were preformed with sera from monkeys collected 2 weeks after their third vaccination (day 84) with the RSSEV DNA vaccine, the CEEV DNA vaccine, both DNA (RSSEV + CEEV) vaccines, the commercial formalin-inactivated CEEV vaccine, or control plasmid DNA containing no foreign gene insert (Control DNA). The cutoff of the assay was the mean OD value of negative control sera plus three standard deviations rounded up to the nearest tenth. The titer was equal to the reciprocal of the last dilution that was above or equal to the OD cutoff value.

RESULTS

Antigenicity of the DNA vaccines and the inactivated CEEV vaccine in rhesus macaques

Groups of four rhesus macaques were vaccinated on days 0, 30, and 70 by gene gun inoculation of DNA vaccines expressing the *prM* and *E* genes of RSSEV, CEEV, or both DNA vaccines. Four control monkeys received the DNA vector plasmid with no insert. Monkeys receiving the individual RSSEV or CEEV vaccines and the control monkeys were given inoculations at four sites, each consisting of 2.5 μg of DNA coated onto 0.5 mg of gold beads. Monkeys that received both vaccines were inoculated at eight sites (four sites with each vaccine) at the same dosage. Four additional monkeys were vaccinated at the same times by intramuscular injection of the human dose (0.5 ml, $\geq$1.0 μg of CEEV antigen) of a commercially available TBE vaccine (FSME-Immun Inject; Immuno-AG, Vienna, Austria). This vaccine consists of formalin-inactivated CEEV formulated with adjuvant. Antibody responses to RSSEV and CEEV were measured by ELISA before each boost and 14 days after the final vaccination. After the first vaccination, none of the monkeys in either the DNA or the commercial vaccine groups displayed detectable antibodies to either RSSEV or CEEV, and after the second vaccination, only low levels were detected (1:50–1:100) (data not shown). Two weeks after the third vaccination, all monkeys had ELISA titers of $\geq$1:100 when screened on RSSEV antigen-coated plates, and all except one had titers when screened on CEEV antigen-coated plates (Fig. 1).

Because ELISA was performed with crude infected-cell lysate as antigen, it is likely that unequal concentrations of RSSEV and CEEV were used to coat the ELISA plates. Therefore, the titers to RSSEV and CEEV cannot be compared directly. Nevertheless, it is clear that the antibody responses obtained with the individual RSSEV or CEEV vaccines were cross-reactive for RSSEV and CEEV antigen. All of the vaccinated monkeys had measurable antibody responses to RSSEV, and all except one (H7T, vaccinated with RSSEV DNA) had responses to CEEV. Compared with the groups of monkeys that received only RSSEV or CEEV DNA, monkeys that received both DNA vaccines had better responses to RSSEV but not to CEEV (Fig. 1). The highest ELISA Geometric mean titer (GMT) to CEEV was obtained with the inactivated vaccine and the

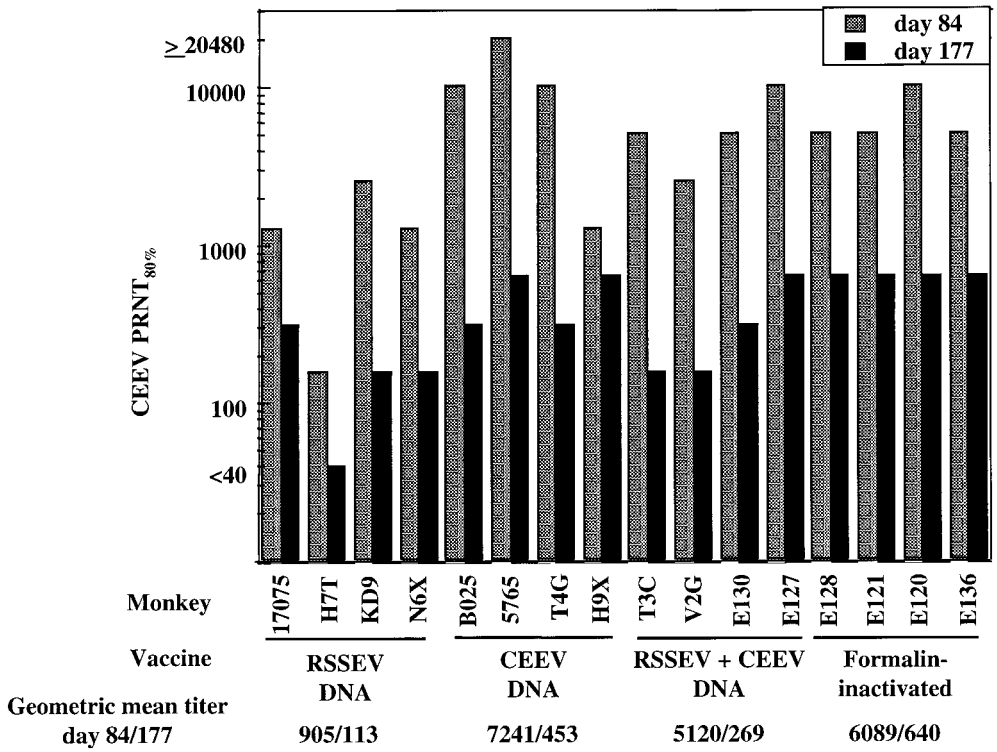

FIG. 2. Neutralizing antibody responses to CEEV were measured for monkey serum samples collected 2 weeks after their third vaccination (day 84) with the RSSEV DNA vaccine, the CEEV DNA vaccine, both DNA (RSSEV + CEEV) vaccines, the commercial formalin-inactivated CEEV vaccine, or control plasmid DNA containing no foreign gene insert (Control DNA). Twofold dilutions of sera (1:20–1:640) were mixed with infectious CEEV and then incubated at 37°C for 1 h and stored at 4°C overnight. The next day, the samples were applied to monolayers of Vero E6 cells, adsorbed for 1 h, and then overlaid. Plaques were visualized by staining with neutral red 5 days later. The neutralizing antibody titer was calculated as a reciprocal of the highest dilution resulting in a 80% reduction of plaques compared with a control of virus with no added antibody ($PRNT_{80\%}$).

CEEV DNA vaccine, with only a twofold difference (Fig. 1).

Neutralizing antibody responses of vaccinated monkeys

Neutralizing antibodies correlate with protective immunity to tick-borne flaviviruses, as demonstrated in mice by passive transfer of neutralizing monoclonal antibodies to *M* and *E* (Holzmann *et al.,* 1997; Heinz *et al.,* 1983). In humans, the administration of commercially available immunoglobulin is recommended for preventing TBE when administered within 96 h after an infected tick-bite (Immuno-AG, 1989). We measured the neutralizing antibody responses to CEEV in monkey serum samples collected 2 weeks after the third vaccination (day 84). All except one of the vaccinated monkeys (H7T) had plaque reduction neutralization titers ($PRNT_{80\%}$) to CEEV of ≥1280 (Fig. 2). Monkeys that received the RSSEV DNA had GMT of neutralizing antibodies to CEEV at least fivefold lower than those that received the other vaccines. The GMT of sera from monkeys that received the CEEV DNA (alone or in combination with RSSEV DNA) and monkeys that received the inactivated vaccine were similar (Fig. 2).

To assess the persistence of the neutralizing antibody responses, blood samples were collected 107 days after the final vaccination (day 177) and again assayed by PRNT to CEEV. Reductions were observed in neutralizing antibody levels of individual monkeys from as little as twofold to more than 30-fold. Nevertheless, $PRNT_{80\%}$ titers for all monkeys except the one that originally had a poor response remained at ≥160 (Fig. 2).

Passive protection of mice by transfer of sera from monkeys vaccinated with the RSSEV and CEEV DNA vaccines

Because neither RSSEV nor CEEV consistently causes acute disease in monkeys, we were unable to evaluate protective efficacy of the DNA vaccines by direct challenge of the primates. However, the known ability of passively transferred immune globulin to protect humans from TBE (Immuno-AG 1989) suggests that protection can be assayed by passive transfer of sera from the immunized monkeys to a susceptible animal. Consequently, we assayed the ability of sera from the vaccinated monkeys to passively protect mice from challenge. For these studies, we administered one additional booster with each vaccine. For the DNA vaccines, we tested two different methods of boosting. One involved a gene gun inoculation at a single site of 2.5 μg of DNA on

TABLE 1

Passive Transfer of 50 or 10 μl of Immune Monkey Sera to Mice and Challenge of Mice with CEEV

Monkey	DNA vaccine	Gene gun boost method[a]	DNA in boost (μg)	RSSEV $PRNT_{50\%}$ monkey serum[b]	CEEV $PRNT_{50\%}$ monkey serum[b]	Serum transfer[c] (μl)	RSSEV $PRNT_{50\%}$ of mice (a, b) 1 h after transfer[d]	CEEV $PRNT_{50\%}$ of mice (a, b) 1 h after transfer[d]	Challenge of mice with CEEV[e]	
									Morbidity	Mortality
T3C	RSSE + CEE	1 site	5	10,240	10,240	50	20, 40	40, 40	0/5	0/5
						10	NT	NT	0/5	0/5
V2G	RSSE + CEE	1 site	5	1280	5120	50	<20, 20	<20, 20	0/5	0/5
						10	NT	NT	2/5	2/5
E130	RSSE + CEE	4 sites	20	≥20,480	≥20,480	50	40, 40	40, 80	0/5	0/5
						10	NT	NT	1/5	1/5
E127	RSSE + CEE	4 sites	20	10,240	≥20,480	50	<20, <20	20, 40	0/5	0/5
						10	NT	NT	1/5	1/5
V9D	Control	4 sites	10	<20	<20	50	<20, <20	<20, <20	5/5	5/5
						10	NT	NT	5/5	5/5

[a] Two different gene gun boost methods were used. The one-site method consisted of one inoculation with RSSEV DNA and one inoculation with CEEV DNA, which delivered 0.5 mg of gold coated with ~2.5 μg of DNA per site at 400 psi, without the spinner hardware modification of the gene gun. The four-site method consisted of four inoculations each of RSSEV or CEEV DNA, which delivered 1 mg of gold coated with ~2.5 μg of DNA per site at 550 psi, with the spinner hardware modification to the gene gun.

[b] Sera from monkeys vaccinated with RSSEV and CEEV DNA, or with a control plasmid were collected 20 days after the final booster vaccination and assayed by plaque reduction neutralization tests. Titers are expressed as the highest reciprocal dilution that reduced the number of plaques by >50% ($PRNT_{50\%}$) compared with controls with no monkey serum.

[c] Monkey sera (50 μl or 10 μl) were diluted to a final volume of 0.1 ml in PBS and were inoculated s.c. into mice.

[d] Two mice (designated a or b) from the 50-μl groups were exsanguinated 1 h after transfer, and $PRNT_{50\%}$ was determined.

[e] Mice were challenged by intraperitoneal injection of 100 LD_{50} of CEEV 1 h after transfer of monkey sera.

NT = not titered.

0.5 mg of gold beads delivered at 400 psi. The second method consisted of gene gun inoculations at four sites for each DNA vaccine. Each site received 2.5 μg of DNA coated onto 1 mg of gold. These injections were given at 550 psi with a gene gun hardware modification that delivered the gold more evenly over the inoculation site. We measured the neutralizing antibody responses of the monkeys at 20 days after the booster inoculation. The GMT ($PRNT_{80\%}$) of all monkeys vaccinated with one booster was 1613 for RSSEV and 2032 for CEEV. For monkeys that received DNA at four sites, the GMT was 2032 and 4561 for RSSEV or CEEV, respectively. The modified gun and protocol, therefore, did not noticeably improve the efficacy of boosting.

To assay protection, we transferred sera from monkeys vaccinated with both DNA vaccines or with the control plasmid to groups of mice by subcutaneous (s.c.) injection. Mice received either 50 μl (approximately 2.5 ml/kg) or 10 μl (approximately 0.5 ml/kg) of monkey sera (diluted in PBS to a final volume of 0.1 ml) 1 h before they were challenged with 100 LD_{50} of RSSEV or CEEV. If all of the transferred serum appeared as circulating neutralizing antibodies in the mice, we calculated that the mice receiving 50 μl of serum should have PRNT titers approximately 20-fold less than those observed in the monkeys and that mice that received 10 μl should have PRNT approximately 100-fold less. However, analysis of blood samples of two mice in each of the 50-μl transfer groups at 1 h after transfer (i.e., immediately before challenge) revealed that their actual neutralizing antibody titers were at least 10 times less than the predicted titers and in some cases were undetectable (Table 1). Despite these low levels of circulating neutralizing antibodies, all five mice that received 50 μl of passively transferred monkey sera remained healthy after challenge with RSSEV or CEEV. Although we did not measure circulating levels of neutralizing antibodies in mice that received 10 μl of monkey sera, we assumed that the actual titer was also less than the predicted titer and was likely to be undetectable by PRNT. Nevertheless, all of the mice in one of the groups remained healthy, and at least three mice remained healthy in the other two groups (Table 1). All mice that died showed delayed times to death compared with control mice (Fig. 3).

Comparison of passive protection of mice by transfer of sera from monkeys vaccinated with the individual DNA vaccines, both DNA vaccines, or the inactivated-virus vaccine

We performed another passive transfer experiment in mice to compare protection elicited by the individual RSSEV or CEEV DNA vaccines with that elicited by both DNA vaccines or the inactivated-virus vaccine. For this study, groups of five mice each received 10 μl of serum from vaccinated monkeys or control monkeys by the s.c. route and were challenged 1 h later with 100 LD_{50} of CEEV or RSSEV.

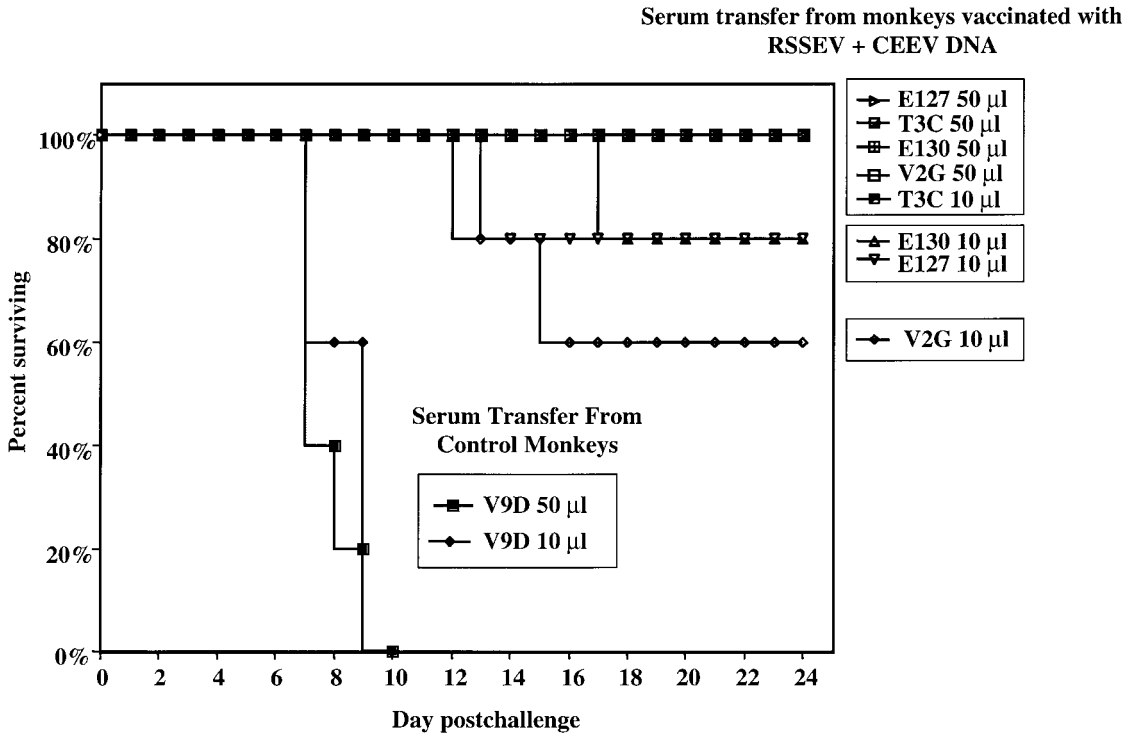

FIG. 3. Passive protection of mice with sera from monkeys immunized with the RSSEV and CEEV DNA vaccines. Groups of 5 mice were injected subcutaneously with 50 μl of serum (diluted to a final volume of 0.1 ml) from individual monkeys vaccinated with both the RSSEV DNA vaccine and CEEV DNA vaccines or with sera from monkeys vaccinated with a DNA plasmid with no foreign gene insert (control monkeys). One hour after serum transfer, the mice were challenged by intraperitoneal injection of approximately 100 LD_{50} of CEEV. Mice were observed daily for morbidity, and the day of death was recorded.

All mice that received sera from monkeys given both the RSSEV and CEEV vaccines remained healthy after RSSEV challenge, and all except two remained healthy after CEEV challenge (Table 2). The two mice that died were recipients of sera from the monkey with the lowest neutralizing antibody response to both RSSEV and CEEV that had received boosters at only one site with 5 μg of DNA (monkey V2G, Table 2). Similarly, all mice that received serum from monkeys that were vaccinated with the formalin-inactivated vaccine remained healthy after RSSEV and CEEV challenge (Table 2).

Of the mice that received sera from monkeys vaccinated with the RSSEV DNA vaccine, all except one remained healthy after RSSEV challenge. Sera from two of the RSSEV DNA-vaccinated monkeys (KD9 and N6X, Table 2) were also able to passively protect all mice from CEEV challenge. However, mice that received sera from the other two monkeys vaccinated with the RSSEV DNA (17075 and H7T, Table 2) were not completely protected from challenge with CEEV. As expected, mice that received monkey sera with the poorest neutralizing activity to CEEV (i.e., serum from monkey H7T) showed the least protection from CEEV challenge (Table 2).

Sera from two monkeys that received the CEEV DNA vaccine (90B025 and T4G, Table 2) passively protected all mice from both RSSEV and CEEV challenge. The serum of another monkey (85765, Table 2) vaccinated with the CEEV DNA protected three of five mice from RSSEV challenge and four of five from CEEV challenge. The final monkey that received the CEEV DNA vaccine (H9X) had very low levels of neutralizing antibodies to RSSEV and CEEV, and its serum failed to protect any mice from morbidity after RSSEV or CEEV challenge (Table 2), although mice that died showed delayed time to death compared with controls (data not shown).

DISCUSSION

Naked DNA vaccines to many viral pathogens have been tested in animal models, but only a few have been evaluated in nonhuman primates (e.g., Fuller *et al.,* 1995, 1996, 1997; Lekutis *et al.,* 1997; Lu *et al.,* 1996; Lu, 1997; Yasutomi *et al.,* 1996) or in humans (e.g., Wang *et al.,* 1998; MacGregor *et al.,* 1998; Lu, 1997). The first clinical study of a DNA vaccine delivered by gene gun particle bombardment was recently concluded. The results demonstrated that three doses of a hepatitis B virus DNA vaccine elicited antibody levels known to correlate with protective immunity. These studies pave the way for further use of gene gun-delivered DNA vaccines for humans.

Gene gun inoculation differs from injection of DNA in that the particle bombardment method can deliver the DNA directly into epidermal cells rather than into intra-

TABLE 2

Passive Transfer of 10 μl of Immune Monkey Sera to Mice and Challenge of Mice with RSSEV or CEEV

Monkey	Vaccine	Gene gun boost method[a]	DNA in boost (μg)	Neutralizing antibody responses of monkeys to RSSEV[b]		Neutralizing antibody responses of monkeys to CEEV[b]		Passive protection in mice: RSSEV challenge[c]		Passive protection in mice: CEEV challenge[c]	
				$PRNT_{50\%}$	$PRNT_{80\%}$	$PRNT_{50\%}$	$PRNT_{80\%}$	Morbidity	Mortality	Morbidity	Mortality
DNA											
17075	RSSE	1 site	2.5	10,240	2560	2560	1280	0/5	0/5	2/5	2/5
H7T	RSSE	1 site	2.5	1280	<640	<640	<640	1/5	1/5	5/5	4/5
KD9	RSSE	4 sites	10	5120	2560	5120	2560	0/5	0/5	0/5	0/5
N6X	RSSE	4 sites	10	10,240	2560	5120	2560	0/5	0/5	0/5	0/5
B025	CEE	1 site	2.5	5120	<640	10,240	5120	0/5	0/5	0/5	0/5
5765	CEE	1 site	2.5	5120	640	10,240	5120	2/5	2/5	1/5	1/5
T4G	CEE	4 sites	10	≥20,480	2560	≥20,480	10,240	0/5	0/5	0/5	0/5
H9X	CEE	4 sites	10	<640	<640	2560	1280	5/5	4/5	5/5	5/5
T3C	RSSE + CEE	1 site	5	10,240	2560	10,240	2560	0/5	0/5	0/5	0/5
V2G	RSSE + CEE	1 site	5	1280	10,240	5120	2560	0/5	0/5	2/5	2/5
E130	RSSE + CEE	4 sites	20	≥20,480	5120	≥20,480	10,240	0/5	0/5	0/5	0/5
E127	RSSE + CEE	4 sites	20	10,240	2560	≥20,480	10,240	0/5	0/5	0/5	0/5
Formalin-inactivated											
E128	Immuno			5120	2560	5120	2560	0/5	0/5	0/5	0/5
E121	Immuno			2560	640	5120	2560	0/5	0/5	0/5	0/5
E120	Immuno			5120	1280	5120	2560	0/5	0/5	0/5	0/5
E136	Immuno			10,240	5120	5120	2560	0/5	0/5	0/5	0/5
Plasmid with no insert											
V9D	Control	4 sites	10			<20	<20	5/5	5/5	5/5	5/5
V3J	Control	4 sites	10			<20	<20	5/5	4/5	5/5	5/5
E126	Control	1 site	2.5			<20	<20	5/5	4/5	5/5	5/5
PBS								20/20	18/20	10/10	9/10

[a] Two different gene gun methods were used for boosting the monkeys. The one-site method consisted of a single gene gun inoculation of each DNA vaccine for monkeys that received only RSSEV or CEEV. For monkeys that received both vaccines, one inoculation of each vaccine was given. Each inoculation consisted of ~0.5 mg of gold coated with ~2.5 μg of DNA and was delivered at 400 psi without the spinner hardware modification of the gene gun. The four-site method consisted of four gene gun inoculations of the individual vaccine, or four of each vaccine for monkeys that received both RSSEV and CEEV DNA. Each inoculation imparted approximately 1 mg of gold coated with ~2.5 μg of DNA at each site and was delivered at 550 psi, with the spinner hardware modification to the gun.

[b] Sera from monkeys vaccinated with RSSEV and CEEV DNA, or with a control plasmid, were collected 20 days after the final booster vaccination and assayed by plaque reduction neutralization tests. Titers are expressed as the highest reciprocal dilution that reduced the number of plaques by >50% ($PRNT_{50\%}$) or 80% ($PRNT_{80\%}$) compared with controls with no monkey serum.

[c] Monkey sera (50 or 10 μl) were diluted to a final volume of 0.1 ml in PBS and were inoculated subcutaneously into mice. One hour after transfer, mice were challenged by intraperitoneal injection of 100 LD_{50} of RSSEV or CEEV.

cellular spaces. Consequently, only small amounts of DNA are required to elicit immune responses. Our previous studies with TBE DNA vaccines in mice demonstrated that DNA expressing the *prM* and *E* genes of RSSEV or CEEV is able to induce neutralizing antibodies and confer cross-protective immunity to RSSEV and CEEV. Moreover, the protective immunity lasted for many months after vaccination (Schmaljohn *et al.,* 1997).

To expand on those studies and as a prelude to possible clinical trials of our DNA vaccines, we evaluated their antigenicity and immunogenicity in monkeys. There is no known satisfactory disease model for TBE in monkeys. Studies performed in bonnet monkeys (*Macaca radiata*) demonstrated disease on infection with one member of the TBE virus complex, Kyasanur Forest disease virus (Kenyon *et al.,* 1992; Hambleton *et al.,* 1983), but these monkeys did not develop disease consistently when infected with RSSEV or CEEV (R. Kenyon, unpublished information). Similarly, only subclinical infections of rhesus macaques (*Macaca mulatta*) were observed after intravenous inoculation of a Turkish strain of a TBE complex virus, and only about half of the animals infected showed neurological disease after intranasal inoculation (Hambleton *et al.,* 1983). Rhesus macaques, therefore, appear to be similar to humans in that only some infected individuals develop encephalitis or other neurological symptoms. Although the monkeys were not a reliable disease model, they developed high levels of neutralizing antibodies after infection and thus were useful as an infection model for evaluating the safety and efficacy of the Austrian inactivated-virus vaccine (Hambleton *et al.,* 1983).

In our studies, we demonstrated that high levels of neutralizing antibodies to RSSEV and CEEV could be obtained with either of the DNA vaccines and with a combination of the two vaccines. After three vaccinations, neutralizing antibody titers to CEEV were high for all groups except those that received only the RSSEV DNA vaccine. It is noteworthy that the CEEV DNA vaccine elicited antibody titers as high as those of the commercial vaccine, even though the inactivated vaccine was given at the human dose to animals that are much smaller than humans. Although we did not measure neutralizing antibody levels to RSSEV after the initial three-dose series, we did measure them for serum samples used for passive transfer studies, which were collected 2 weeks after an additional (fourth) booster inoculation. As a group, we found that the monkeys vaccinated with both DNA vaccines developed higher levels of neutralizing antibodies to RSSEV ($PRNT_{80\%}$ = GMT 4305) than those that received only the RSSEV DNA vaccine (GMT 2153), the CEEV DNA vaccine (GMT 640), or the commercial inactivated CEEV vaccine (GMT 1810). Likewise, as a group, monkeys vaccinated with both DNA vaccines had higher levels of neutralizing antibodies to CEEV ($PRNT_{80\%}$ = GMT 5120) than did monkeys vaccinated with the RSSEV DNA vaccine (GMT 1280), the CEEV DNA vaccine (GMT 4305), or the commercial inactivated-virus vaccine (GMT 1280). For the DNA vaccines, we do not know whether the higher titers reflect a broadened immune response elicited by the combination of the two vaccines compared with the individual vaccine or if they reflect the influence of twice as much DNA distributed over twice as many sites on the skin per vaccination. Future studies aimed at determining optimal dosages of the DNA vaccines may address this question. Regardless of the reason, the combination of both DNA vaccines generated neutralizing antibody responses quantitatively equal to those elicited by a vaccine known to protect from TBE. In addition, the antibody titers persisted for at least 15 weeks after vaccination at levels well above what we demonstrated to offer complete protection in mice.

Not only were antibody titers similar in monkeys vaccinated with both DNA vaccines or with the inactivated-virus vaccine, but also the protective immunity, as measured by passive transfer of serum from monkeys to mice, also appeared to be very similar. That is, all 20 mice in groups that received sera from monkeys vaccinated with both DNA vaccines or in groups that received sera from monkeys vaccinated with the inactivated-virus vaccine remained healthy after RSSEV challenge. Although 2 of the 20 mice given sera from monkeys vaccinated with both DNA vaccines did not survive challenge with CEEV, whereas all 20 that received sera from monkeys vaccinated with the inactivated-virus vaccine remained healthy, this difference was not found to be statistically significant. Of course, it is possible that the inactivated-virus vaccine elicits a broader and more effective immune response because of the additional antigens in the vaccine; however, further studies are required to address that possibility.

In summary, these studies offer encouragement for the use of DNA vaccines for TBE in humans. Additional studies will be required to determine optimal parameters of dosage and frequency of vaccination required to elicit protective immunity in humans.

MATERIALS AND METHODS

Viruses, cells, and media

The origins of the RSSEV, strain Sofjin, and CEEV, strain Hypr, were described previously (Schmaljohn *et al.,* 1997; Calisher 1988). RSSEV and CEEV were propagated and assayed in Vero E6 cells maintained in Eagle's minimal essential medium supplemented with 10% FBS and antibiotics as described previously (Schmaljohn *et al.,* 1997). All studies with infectious RSSEV or CEEV were conducted in a biosafety level 4 laboratory.

Preparation of gene gun cartridges

Construction of the DNA vaccines expressing the *prM* and *E* genes of RSSEV and CEEV were described previously (Schmaljohn *et al.,* 1997). Plasmid DNA was precipitated onto the outside surface of gold beads (approximately 2 μm in diameter) as described earlier (Eisenbraun *et al.,* 1993). The DNA-coated gold particles were dried on the inside walls of Tefzel tubing, which was then cut into 0.5-inch sections to make cartridges for the gene gun (Pertmer *et al.,* 1995). Final amounts of DNA in each cartridge was measured by fluorometric assays of eluted DNA or estimated by gel electrophoresis and comparison with ethidium bromide-stained standards.

Monkey vaccinations

Monkeys were anesthetized with Telazol at a dosage of 3–6 mg/kg body wt. before handling, vaccination, and phlebotomy. The PowderJect-XR gene delivery device (D. McCabe, inventor; Agracetus, Inc., assignee; PCT patent WO 95/19799, July 27, 1995) was used to achieve intracellular inoculation of epidermal cells. Twenty adult rhesus macaques (*Macaca mulatta*) were randomized into five groups of four animals each. Groups 1–4 received naked DNA vaccines for RSSEV, CEEV, RSSEV and CEEV, or plasmid with no insert, respectively. DNA vaccinations consisted of gene gun inoculation of approximately 2.5 μg of DNA coated onto 0.5 mg of gold beads delivered to the lower abdomen at each of four sites (for the monkeys receiving the individual DNA vaccines) or eight sites (for the monkeys receiving both DNA vaccines) with a pressure setting of 400 psi. Group 5 monkeys received a European commercial vaccine for TBE (FSME-Immun Inject; Immuno AG). This vaccine is a suspension of

purified CEEV that is propagated in chicken embryo cells, inactivated with formalin, and formulated with aluminum hydroxide adjuvant. Monkeys received the vaccine at the recommended human dose of 0.5 ml intramuscularly in the upper arm using the preloaded needle and syringe that accompany the vaccine. All vaccines were administered on days 0, 30, and 70. Blood samples were collected before each vaccination and at days 84 and 177. Samples from days 0, 30, 70, and 84 were examined by ELISA, and samples from days 84 and 177 were assayed by PRNT. At day 177, a booster inoculation of each vaccine was administered. For the commercial vaccine, the same dosage was used as in earlier vaccinations. For the DNA vaccines, two monkeys in each group received approximately 2.5 μg of the RSSEV vaccine, the CEEV vaccine, or the control plasmid at a single site on the lower abdomen (two sites for those that received both the RSSEV and CEEV vaccines). The other two monkeys in each group received approximately 2.5 μg of gold delivered to each of four sites (eight sites for those that received both vaccines). For these monkeys, a modified gene gun protocol was used in which a hardware modification of the gun ("spinner" insert) was used that was intended to result in more even distribution of the gold over the inoculation site. In addition, the pressure was increased from 400 to 550 psi and the amount of gold was increased from 0.5 to 1 mg/dose. Blood samples were collected 20 days after the vaccination and assayed by PRNT.

Serological assays

Direct IgG ELISA was performed as described earlier (Schmaljohn *et al.,* 1997). The cutoff of the assay was the mean OD value of negative control sera plus three 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. PRNT assays were performed as previously described (Schmaljohn *et al.,* 1997).

Passive transfer of immune sera to mice and challenge with RSSEV or CEEV

Monkey sera were diluted in sterile PBS to deliver 50 μl (~2.5 ml/kg) or 10 μl (~0.5 ml/kg) in a volume of 0.1 ml. Each diluted serum was injected subcutaneously into 6- to 8-week-old BALB/c mice. The blood volume of a BALB/c mouse is approximately 5.85 ml/100 g. The 6- to 8-week-old female BALB/c mice in our experiments weighed approximately 20 g. Therefore, each mouse had a blood volume of approximately 1.17 ml. Passive transfer of 10 or 50 μl of monkey sera to a BALB/c mouse resulted in a calculated dilution of approximately 1:117 or 1:23, respectively. One hour after transfer, the mice were challenged by intraperitoneal inoculation of approximately 50 PFU of suckling mouse brain-passaged RSSEV or CEEV, a dose previously determined to be approximately 100 LD_{50} for BALB/c mice. Mice were observed daily for 25 days for signs of illness and for death.

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