Immunogenicity of a Japanese encephalitis DNA vaccine candidate in cynomolgus monkeys

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Abstract

A Japanese encephalitis (JE) vaccine candidate encoding JE virus premembrane (prM) and envelope (E) genes, designated pNJEME, was evaluated for safety and immunogenicity in non-human primate, cynomolgus monkeys. pNJEME was constructed using a vector (pNGVL4a) designed to address some of the safety concerns of DNA vaccine. In two different experiments, two immunizations with 300 μg of pNJEME by intramuscular (i.m.) injection, and 3 μg of pNJEME using a gene gun, and three immunizations by i.m. injection with 500 μg of pNJEME were performed. All the three protocols induced low to high levels of neutralizing antibody, indicating an ability of pNJEME to induce neutralizing antibody in monkeys with a wide individual variation in response to pNJEME. In one experiment designed to compare the DNA vaccine with a commercial inactivated JE vaccine, three immunizations by i.m. inoculation with 300 μg of pNJEME or by gene gun administration with 3 μg of pNJEME induced similar levels of neutralizing antibody to those induced by three immunizations with a human dose of the inactivated vaccine in most monkeys. After intranasal challenge with the Beijing P3 or JaTH160 strain of JE virus, pNJEME-immunized monkeys showed anamnestic neutralizing antibody responses, indicating that pNJEME induced memory B cells which were responsive to infection with JE virus. No systemic and local reactions were observed in any monkeys after i.m. or gene gun inoculations with plasmid DNAs.

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1. Introduction

The genus Flavivirus consists of about 70 viruses, of which 50 are transmitted by mosquitoes or ticks [1]. More than 30 arthropod-borne flaviviruses cause human diseases including those of global importance, such as dengue, yellow fever (YF), tick-borne encephalitis (TBE) and Japanese encephalitis (JE). Licensed vaccines are currently available for human use only against some of these flavivirus diseases; inactivated vaccines for TBE and JE and attenuated vaccines for YF. However, there are no vaccines available for dengue and other arthropod-borne flaviviruses of regional concern including West Nile (WN), St. Louis encephalitis (SLE), and Murray valley encephalitis (MVE) viruses.

In the last 10 years, DNA vaccines have been studied for a variety of infectious diseases [2–4]. This approach has a strategy that a portion of the genes of a pathogenic agent incorporated in a plasmid vector is expressed in the host, allowing the host to induce protective immunity. Based on this strategy, DNA vaccines have advantages over existing vaccines in cost and easiness for production, and they also are considered to be a promising approach to development of vaccines which are currently unavailable.

DNA vaccine candidates against several flaviviral diseases have been developed. Studies using dengue type 1 [5], dengue type 2 (6–8), TBE [9,10], JE [11–17], WN [18,19], SLE [20], MVE [21] and louping ill [22] viruses demonstrated the ability of DNA vaccines to induce virus-specific immune responses and/or protection from lethal challenge in mice. Only dengue type 1 [23,24] and TBE [25] DNA vaccine candidates have been evaluated in non-human primates. A TBE DNA vaccine candidate induced neutralizing antibody in rhesus macaques [25], whereas a dengue
type I DNA vaccine candidate induced neutralizing antibody and provided partial protection from viremia following challenge, in thalus macaques [23] and Aotus monkeys [24].

Premembrane (prM), envelope (E) and nonstructural 1 (NS1) proteins are considered to be immunogenic, based on passive protection studies using mice [26–32]. We have selected the prM and E genes for constructing JE vaccine plasmids [33–35], since the prM/E gene cassette has an ability to produce an excellent immunogen in mammalian cells [36,37]. A pCMV-based JE DNA vaccine candidate (designated pcJEME) induced neutralizing antibody in mice and provided protection from lethal challenge [33]. This plasmid also induced neutralizing antibody in swine, an amplifier of JE virus [35]. In order to address some of the safety concerns of DNA vaccine use, we also constructed another DNA vaccine (designated pNJEME) based on pNGVL4a, which utilizes a kanamycin-resistance gene and does not contain any SV40-derived sequences. The ability of pNJEME to induce neutralizing antibody in mice and swine was equivalent to that of pcJEME, which contains an ampicillin-resistance gene and SV40 DNA [35].

In the present study, we evaluated pNJEME for safety and immunogenicity in non-human primate, cynomolgus monkeys. The results indicated that two or three immunizations with pNJEME did not cause any detectable local and systemic reactions and induced specific antibodies and JE virus-responsive memory B cells in monkeys.

2. Materials and methods

2.1. Plasmids

Construction of pNJEME has been previously described [35]. Briefly, the JE virus (Nakayama strain) signal sequence of prM, prM and E genes was cloned from pcJEME [33] into the pNGVL4a vector and resequenced. The pNGVL4a vector contains a eukaryotic promoter derived from human cytomegalovirus with the intron A sequence, a polyadenylation signal, and an immunostimulatory sequence on the modified ampicillin-resistance gene, but not SV40-derived sequences. The pNJEME plasmid did not contain any SV40-derived sequences, and pNJEME was renamed as pUMVC4a. All the plasmid DNAs (pNJEME, pNGVL4a) were purified using a Qiagen endotoxin-free DNA purification kit (EndoFree Plasmid Mega Kit; Funakoshi Co. Ltd., Tokyo, Japan).

2.2. Viruses

The Nakayama strain of JE virus [33] was used for neutralization tests. The Beijing P3 [33] and JaTHI160 [38] strains of JE virus in the form of an infected mouse brain homogenate at a 10% emulsion in 7.5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) were used for challenge studies. Virus titers contained in the homogenate was approximately \(2 \times 10^7\) PFU/ml for the P3 and JaTHI160 strains.

2.3. Animals

Four- to 20-year-old male and female cynomolgus monkeys, Macaca fascicularis, bred in the Tsukuba Primate Center for Medical Science, with body weights ranging from 2.3 to 10.6 kg, were used. Before handling, bleeding, immunization, and challenge, monkeys were anesthetized with 50 mg/ml Ketamine (Ketalar 50; Sankyo, Tokyo, Japan) at a dosage of 0.2 ml/kg (10 mg/kg). All animals had no history of flavivirus infection and no detectable neutralizing antibody and hemagglutination-inhibiting antibody prior to use. The monkeys were kept in individual cages (45 cm in width, 60 cm in depth, 60 cm in height) at 25 ± 2 °C under the light (12 h) and dark (12 h) protocol, and fed 100 g of apples and 70 g of pelleted diet once a day. All animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals in the National Institute of Infectious Diseases under approval of the animal research committee of National Institute of Infectious Diseases.

2.4. Immunization and challenge

Animals were immunized three times at intervals of 4–6 weeks by intramuscular (i.m.) inoculation with 300 or 500 μg of pNJEME or by inoculation with 3 μg of pNJEME using a gene gun. The i.m. injection was performed using a 1 cm\(^3\) syringe with a 26-gauge needle and the plasmid DNAs diluted in PBS were injected at two sites in the quadriceps muscle with a volume of 0.5 ml per site. The gene gun inoculation was done using Helios Gene Gun System (Bio-Rad, Hercules, CA) with 0.5 μg of DNA coated onto 0.25 mg of gold particles with a diameter of 1.6 μm. The plasmid DNAs were delivered to the skin of both thighs at each of six sites (three sites per thigh) with a pressure setting of 400 psi (28 kg/cm\(^2\)). All animals had no history of flavivirus infection and no detectable neutralizing antibody and hemagglutination-inhibiting antibody prior to use. The monkeys were kept in individual cages (45 cm in width, 60 cm in depth, 60 cm in height) at 25 ± 2 °C under the light (12 h) and dark (12 h) protocol, and fed 100 g of apples and 70 g of pelleted diet once a day. All animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals in the National Institute of Infectious Diseases under approval of the animal research committee of National Institute of Infectious Diseases.

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For i.n. inoculation, 0.25 ml of the virus solution were administered into each of the right and left nasal passages with a 1 cm\(^3\) syringe without needle. Since our i.n. challenge system did not constantly cause encephalitis in monkeys, we used the challenge mainly to examine anamnestic responses to the infection. At the end of the observation period, or during the observation period if severe symptoms of encephalitis were developed, monkeys were euthanized. Sera were collected from monkeys at 1- to 4-week intervals after immunization and at 1-week intervals after challenge. For a control, monkeys were vaccinated with one human dose of a commercial inactivated JE vaccine three times at 2-week intervals by subcutaneous (s.c.) injections.
2.5. Neutralization test

Neutralizing antibodies were determined using plaque reduction assays performed with the Nakayama strain in the presence of rabbit complement (Cedarlane Laboratories, Hornby, Canada) at the final concentration of 5% in antigen-antibody mixture based on the method described previously [36]. In most cases, the neutralizing antibody titer was expressed as the maximum serum dilution yielding a 90% reduction in plaque number, and in other cases a 70% plaque reduction was used as a borderline to express a 90% reduction in plaque number, and in other cases a borderline to express the titer. We adopted a 90% reduction, since we have previously used this borderline for evaluation of the same DNA vaccine (pNJEME) in mice and swine [35].

2.6. Enzyme-linked immunosorbent assay (ELISA) for quantification of antibodies to E

Anti-E antibodies in monkey sera were quantified by a conventional ELISA essentially as previously described [37]. Briefly, microplates (Microwell, Nunc, Roskilde, Denmark) were sensitized with JE virus E antigen at 30 μg per well. The E antigen was produced from a cell line continuously expressing extracellular particles (EPs) and purified from culture fluids by polyethylene glycol precipitation and sucrose density gradient centrifugation. Sensitized plates were incubated serially with a 1:100 dilution of test sera, a 1:300 dilution of alkaline phosphatase-conjugated anti-monkey IgG (heavy and light chain-specific: Bethyl Laboratories), monkey IgG (heavy and light chain-specific: Bethyl Laboratories) at the final concentration of 5% in the diluent starting from a 1:10 dilution.

Activity and appetite remained normal (data not shown).

3. Results

3.1. Safety

No systemic or local reactions were observed in any monkeys inoculated with plasmid DNAs (pNJEME or pNGVL4a) by the i.m. route or using a gene gun during the period prior to challenge (11 or 12 weeks). Specifically, none of the monkeys showed skin rash at the injection site and there were no significant changes in body weight. Activity and appetite remained normal (data not shown). These results indicate the safety of the DNA vaccine.

3.2. Induction of neutralizing antibody detected by a 90% plaque reduction assay

3.2.1. Experiment 1

Male and female monkeys at the age of 11–19 years with body weights ranging from 3.3 to 10.6 kg were used in Experiment 1 to examine the ability of pNJEME to induce neutralizing antibody. Two or three animals in each group were immunized three times at intervals of 4 and 6 weeks by i.m. injection with 300 μg of pNJEME or pNGVL4a or by gene gun administration with 3 μg of pNJEME. Two weeks after the third immunization, monkeys were challenged i.n. with 1 × 106 PFU of the P3 strain of JE virus, and observed for 4 weeks. All animals were bled at intervals of 1 or 2 weeks and individual sera were examined for neutralizing antibody in a 90% plaque reduction assay. As a control, two monkeys were immunized three times at intervals of 2 weeks with one human dose of JEVAX. This control experiment started 14 weeks earlier than the first immunization of other groups with plasmid DNAs. Thus, these JEVAX-immunized animals received fourth immunization with one human dose of JEVAX 2 weeks prior to P3 virus challenge which was performed simultaneously with other three groups. Time courses of neutralizing antibody titers are shown in Fig. 1.

Two of three monkeys immunized i.m. with pNJEME (Fig. 1A) developed low levels of neutralizing antibody (1:10 and 1:20) 2 weeks after the second immunization and
Following challenge, neutralizing antibody titers increased in all immune monkeys except for those that died (⁄⁄ and ⁄⁄) and those that showed titers of 1:1280 or higher at challenge (⁄⁄ and ⁄⁄). The four- or eight-fold increase in titer was shown within 1–2 weeks after challenge. Although our i.n. challenge system does not seem to induce encephalitis consistently in monkeys, two pNJEME-immunized animals developed neurological symptoms and were euthanized 12 (⁄⁄) and 13 (⁄⁄) days after challenge. One animal that was immunized by i.m. inoculation (⁄⁄) did not show detectable levels of neutralizing antibody in a 90% plaque reduction assay at the time of challenge. However, another animal that was immunized by gene gun inoculation (⁄⁄) had neutralizing antibody titers of 1:20 to 1:40 after challenge until euthanization. Since two non-immune animals did not develop the disease after challenge, there may be wide individual variations in response to the challenge under i.n. inoculation conditions adopted in the present study. Based on the protective role of neutralizing antibody as demonstrated in several experimental models using mice [29,30,34] and monkeys [31], the failure of protection in a neutralizing antibody-positive animal may be attributed to unknown mechanism(s). It is possible that in i.n. challenge neutralizing antibody was not able to work for disease protection or possibly the challenge virus directly penetrated to the brain through the olfactory bulb.

3.2.2. Experiment 2

Groups of four or five 4-year-old male monkeys with body weights ranging from 2.3 to 4.0 kg were immunized three times at intervals of 4 weeks i.m. with 500 H9262 gopNJEME or pNGVL4a, and challenged i.n. with 1 × 10⁹ PFU of the JaTH160 strain of JE virus 3 weeks after the third immunization. Sera were collected from animals at 1- to 2-week intervals until euthanization (mostly 6 weeks after challenge), and were examined for neutralizing antibody in a 90% plaque reduction assay. Postchallenge sera were also tested for NS1 antibody to confirm that the monkeys were infected by the challenge virus. Some of the challenged animals did not develop detectable levels of NS1 antibody after challenge (see Table 1), and time courses of neutralizing antibody were analyzed in animals grouped according to immunogens and the presence of NS1 antibody (Fig. 2). One pNGVL4a-inoculated monkey (⁄⁄), that developed neurological symptoms and euthanized 14 days after challenge, was grouped into animals that did not develop detectable levels of NS1 antibody, but this animal was infected with virus based on the symptom characteristic of JE, different from the other animal (⁄⁄) that was not infected based on the absence of symptoms and NS1 antibody throughout the observation period after challenge (6 weeks).

One of five monkeys immunized with pNJEME (⁄⁄) developed a neutralizing antibody titer of 1:10 after two immunizations and two animals developed titers of 1:20 (⁄⁄) and 1:90 (⁄⁄) 1 week after the third immunization. One of the remaining two animals (⁄⁄) developed an antibody titer of 1:20 three weeks after the third

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Fig. 1. Time courses of neutralizing antibody titers (90% plaque reduction assay) in Experiment 1. Monkeys were immunized with pNJEME by i.m. (A) or gene gun (B) inoculation, pNGVL4a (C) or JEVAX (D). Immunization with JEVAX started 14 weeks earlier than the immunization with DNAs, and all animals were challenged at the same time. Arrow heads indicate the time of immunization, and arrows indicate the time of challenge.
Table 1
NS1 antibody titers in monkeys following challenge in Experiment 2

<table>
<thead>
<tr>
<th>Immunogen</th>
<th>Monkey</th>
<th>NS1 antibody titer*</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNJEME</td>
<td>#25</td>
<td>&lt;1:10 &lt;1:10 &lt;1:10 &lt;1:10 &lt;1:10 1:40</td>
</tr>
<tr>
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<td>&lt;1:10 &lt;1:10 &lt;1:10 &lt;1:10 &lt;1:10 1:40</td>
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<tr>
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<td>#22</td>
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</tr>
<tr>
<td>pNGVL4a</td>
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</tr>
<tr>
<td></td>
<td>#27</td>
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</tr>
<tr>
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</tr>
<tr>
<td></td>
<td>#24</td>
<td>&lt;1:10 &lt;1:10 NA NA NA NA</td>
</tr>
</tbody>
</table>

* Determined by an immunostaining method using NS1-expressing and non-expressing cells as antigens (see Section 2 for details).

Monkeys were inoculated three times with 500 μg of indicated immunogens and challenged i.n. with the JaTH160 strain of JE virus. Five animals immunized with pNJEME and four animals inoculated with pNGVL4a were further grouped according to development of NS1 antibody.

- Weeks after challenge; “−13” indicates 2 weeks prior to the first immunization.
- Not available due to euthanization of the animal.

immunization. The other pNJEME-immunized animal (#28) did not develop detectable levels of neutralizing antibody throughout the immunization period prior to challenge. As expected, neutralizing antibody was not detected in four animals inoculated with pNGVL4a (Fig. 2C and D). These results support the indication obtained in Experiment 1 that pNJEME can induce neutralizing antibody (90% plaque reduction assay) in most of the monkeys.

Comparison of neutralizing antibody titers shown in Experiments 1 and 2 indicates that 300 μg of pNJEME in Experiment 1 was more immunogenic than 500 μg of pNJEME in Experiment 2. This result suggests that there is a difference in the levels of immune responses among monkeys.

Postchallenge time courses of neutralizing antibody titers indicated that pNJEME-immunized monkeys showed more rapid increase than non-immune monkeys after challenge (compare Fig. 2A and C). Specifically, monkeys immunized with pNJEME increased neutralizing antibody to high levels within 2 weeks after challenge, whereas 3–4 weeks were taken to increase neutralizing antibody in pNGVL4a-inoculated monkeys. These results indicate anamnestic responses to the challenge in pNJEME-immunized animals, and that immunization with pNJEME induced memory B cells which were responsive to infecting JE virus.

3.3. Induction of neutralizing antibody detected by a 70% plaque reduction assay and ELISA antibodies

Although two pNJEME-immunized monkeys (#4 and #28) did not develop detectable levels of neutralizing antibody in a 90% plaque reduction assay throughout the experimental period, neutralizing antibody at a titer of 1:10 was detected in a 70% plaque reduction assay in these animals 1 week after the third immunization. All of the pNGVL4a-inoculated monkeys at the same period still showed no detectable levels of neutralizing antibody in a 70% plaque reduction assay (data not shown). To confirm if these animals developed JE virus-specific antibodies, sera were examined for anti-E antibodies in ELISA. Consistent with the result of the 70% plaque reduction neutralization assay.
test, low but detectable levels of ELISA antibody were shown in sera collected from two pNJEME-immunized monkeys (#4 and #28) 1 week after the third immunization and no non-immune animals at the same period showed detectable levels of ELISA antibody (data not shown). These results indicate that three immunizations with pNJEME induced neutralizing antibody detectable in a 70% plaque reduction assay in all monkeys.

3.4. Induction of postchallenge NS1 antibody

Time courses of the appearance of NS1 antibody titters following challenge in Experiment 2 are shown in Table 1. NS1 antibody was first detected 5–6 weeks postchallenge in pNJEME-immunized monkeys (#25, #26), and 4–5 weeks postchallenge in pNGVL4a-inoculated monkeys (#21 and #27). Higher NS1 antibody titers were detected in pNGVL4a-inoculated monkeys than pNJEME-immunized ones 6 weeks postchallenge. Although a small number of animals were used for comparison, pNJEME-immunized monkeys that developed neutralizing antibodies at challenge tended to show longer incubation periods for development of NS1 antibody than pNGVL4a-inoculated animals that did not develop neutralizing antibodies at challenge.

In Experiment 1, the only postchallenge serum which had NS1 antibody was a sample from a JEVAX-immunized monkey (#9) 4 weeks postchallenge, and the titer was 1:80 (data not shown). Since the experimental period following challenge was only 4 weeks in Experiment 1, it is likely that most of the monkeys, even though infected, did not develop detectable levels of NS1 antibody during this period. Therefore, we did not use NS1 antibody as an indicator of infection in Experiment 1.

4. Discussion

DNA vaccine candidates for JE have been constructed with different strategies using NS1 [11], E [12–14] and prM/E [15,16,33] genes and have been demonstrated to confer partial or full protection in mouse models. The NS1 strategy [11] is based on complement-mediated antibody-dependent cytotoxic mechanism as reported with other flaviviruses [40,41], whereas the protection by E is based on non-neutralizing anti-E antibody which may show antiviral activity through activation of complement and Fc receptor-bearing phagocytic cells [14]. On the other hand, the mechanisms of protection conferred by the prM/E genes are considered to be based on the ability of prM/E to induce neutralizing antibody [15,16,33]. The prM/E strategy to induce neutralizing antibody has been reported with DNA vaccine candidates for other flaviviruses, such as dengue type 1 [5], TBE [9,10], WN [18], SLE [20], MVE [21] and louping ill [22] viruses. Recently, we demonstrated that in vivo virus clearance and in vivo neutralization by neutralizing antibody are the most important mechanism of protection from peripheral challenge in prM/E gene-immunized mice (Konishi et al., in submission). Since virus dissemination from the initial infection site to the central nervous system is critical for disease development in human and equine JE, neutralizing antibody in the circulation is an efficient mechanism to prevent the disease. Based on the importance of neutralizing antibody, we mainly analyzed neutralizing antibody titers for the present evaluation of our DNA vaccine in monkeys.

Evaluation of candidate vaccines for safety and immunogenicity using non-human primates are indispensable as a preclinical step. In our previous evaluation using mice and swine [35], two immunizations with pNJEME induced low and moderate levels of neutralizing antibody in 90% plaque reduction assays. Interestingly, immunogenicity of pNJEME in swine was higher than a commercial inactivated JE vaccine for animal use. In the present study, three immunizations with 300 μg of pNJEME induced equivalent levels of neutralizing antibody to three immunizations with one human dose of JEVAX in most monkeys in Experiment 1. However, two monkeys developed neutralizing antibody detectable in a 70% plaque reduction neutralization test and ELISA, but not in a 90% plaque reduction assay, suggesting relatively high levels of individual differences in immune responses among the monkey population. Variations in antibody responses to DNA vaccines among monkey individuals have been shown in studies evaluating DNA vaccine candidates against other flavivirus diseases. In one study with a dengue type 1 DNA vaccine [23], two of eight rhesus macaques immunized three times i.m. with 1 mg of DNA did not develop neutralizing antibody (50% plaque reduction assay), although the remaining six monkeys developed neutralizing antibody in a range of 1:20 to 1:320. Another dengue type 1 DNA vaccine study [24] indicated that three Aotus monkeys immunized three times i.m. with 1 mg of DNA developed neutralizing antibody titers of <1:10, 1:40 and 1:160 (50% plaque reduction assay). In a TBE DNA vaccine study [25], all the usus macaques immunized three times with 2.5 μg of DNA using a gene gun developed detectable levels of neutralizing antibody (80% plaque reduction assay) with relatively high levels of individual variations (≥200-fold differences).

The intracerebral or i.n. routes have been used for challenge to evaluate protective efficacy of recently developed chimeric [42–44] or recombinant virus [45–47] vaccines against JE in monkeys. Although humans are usually infected with JE virus through the s.c. route by infected mosquito bites, monkeys cannot develop the disease following s.c. inoculation of JE virus [48]. We selected the i.n. route for our study, since we considered that the direct inoculation of JE virus into the brain cannot provide an opportunity for the vaccine-induced host immune response to work for protection. However, the i.n. route caused encephalitis in only a small population of the challenged monkeys under the present conditions with the challenge dose of 1 × 10⁹ PFU (0.5 ml of undiluted seed virus).
Therefore, we used i.n. challenge to evaluate the secondary neutralizing antibody responses in monkeys vaccinated with pNJEME, in addition to the observation of clinical signs. Unfortunately, even infection was not always established by the i.n. challenge as demonstrated by the absence of antibody to NS1. Nevertheless, the present study demonstrates that pNJEME has an ability to induce JE virus-responsive memory B cells in monkeys.

Longer incubation periods for development of NS1 antibody in immune (#25 and #26) than non-immune (#21 and #27) animals suggest that neutralizing antibody had a suppressive effect on growth of the challenge virus in i.n.-inoculated monkeys. One pNJEME-immunized monkey (#29) that did not develop detectable levels of NS1 antibody raised postchallenge neutralizing antibody titers by eight-fold and maintained high titers comparable to those obtained in pNJEME-immunized animals that were infected by the challenge virus (#25 and #26). It is possible that NS1 antibody was under development in this animal (#29), but the antibody level was undetectable at the end of the observation period (6 weeks), or that this animal that had high-level neutralizing antibody at challenge achieved sterile immunity against the challenge virus and JE virus protein antigens included in the inoculum (10% homogenate of infected mouse brains) contributed to booster immune responses. On the other hand, another pNJEME-immunized monkey (#28) that did not show detectable levels of neutralizing antibody and NS1 antibody during 6 weeks postchallenge does not seem to have acquired infection by the challenge virus.

In conclusion, this paper reported results of the first evaluation of a JE DNA vaccine in non-human primates. Our JE DNA vaccine candidate, pNJEME, was safe and immunogenic in cynomolgus monkeys. Currently, we are investigating approaches to enhance immunogenicity of pNJEME in monkeys including use of adjuvant and improvement of gene delivery to minimize individual variations in responses to the DNA vaccine.

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References


