Dengue virus type 1 DNA vaccine induces protective immune responses in rhesus macaques

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A candidate DNA vaccine expressing dengue virus type 1 pre-membrane and envelope proteins was used to immunize rhesus macaques. Monkeys were immunized intramuscularly (i.m.) or intradermally (i.d.) by three or four 1 mg doses of vaccine, respectively. Monkeys that were inoculated i.m. seroconverted more quickly and had higher antibody levels than those that were inoculated i.d. The sera exhibited virus-neutralizing activity, which declined over time. Four of the eight i.m.-inoculated monkeys were protected completely from developing viraemia when challenged 4 months after the last dose with homologous dengue virus. The other four monkeys had reduced viraemia compared with the control immunized monkeys. The i.d.-inoculated monkeys showed no reduction in viraemia when challenged with the virus. All vaccinated monkeys showed an anamnestic antibody response, indicating that they had established immunological memory. Vaccine-induced antibody had an avidity index similar to that of antibody induced by virus infection; however, no clear correlation was apparent between antibody avidity and virus neutralization titres.

Introduction

Dengue viruses are positive-sense, single-stranded RNA viruses belonging to the genus Flavivirus, family Flaviviridae (Chambers et al., 1990). The four antigenically distinct serotypes of dengue virus (dengue-1, -2, -3 and -4) cause an estimated 100 million infections, resulting in widespread dengue fever (DF) and at least 250,000 cases of dengue haemorrhagic fever (DHF), each year (Halstead, 1988). These viruses are transmitted primarily by the mosquito Aedes aegypti and are endemic throughout the tropical and sub-tropical regions of the world. In recent years, a major expansion of dengue transmission has occurred throughout the tropical Americas, accompanied by an alarming increase in the number of DHF cases. Despite the public health importance of DF and DHF, effective control measures are not available other than vector reduction, which has proven difficult and costly to sustain over time (Gubler & Trent, 1993). Although vaccines are available to two related flaviviruses, yellow fever (YF) virus and Japanese encephalitis (JE) virus (Barrett, 1997a, b), decades of effort have not produced a successful dengue virus vaccine. Live-attenuated dengue viruses, while immunogenic (Bhamarapravati & Yoksan, 1989), have been associated with unacceptable reactogenicity and clinical complications (Edelman et al., 1994; McKee et al., 1987). Inactivated dengue viruses have been reported to be less immunogenic (Schlesinger, 1977) and recombinant dengue proteins have failed to protect non-human primates from homologous dengue virus challenge (Deubel et al., 1988; Eckels et al., 1994).

We have been exploring DNA vaccine technology, in which plasmids expressing appropriate viral antigens are used for immunization. This approach has been shown by others to induce an immune response in animal models against a number of different viruses, including several flaviviruses. DNA vaccine constructs for Murray Valley encephalitis virus (Colombage et al., 1998), JE virus (Konishi et al., 1998), Russian spring-summer encephalitis virus (Schmaljohn et al., 1997), Central European encephalitis virus (Schmaljohn et al., 1997), and St Louis encephalitis virus (Phillpotts et al., 1996) have all been reported...
to induce antibodies in mice and full or partial protection from live virus challenge. We have shown previously that a dengue-2 DNA vaccine induced an antibody response in mice (Kochel et al., 1997) and that the mice were protected from dengue-2 virus challenge (Porter et al., 1998). Here, we report complete to partial protection of rhesus macaques that were immunized with a dengue-1 DNA vaccine and challenged with virus.

**Methods**

- **Animals.** The experiments reported herein were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, DHHS Publication number NIH-86-23 (1985). Rhesus macaques (Macaca mulatta), aged 7–24 years, were housed at the Walter Reed Army Institute of Research animal facility in Forest Glen, MD, USA. The monkeys were pre-screened for the presence of dengue-specific antibody by ELISA and the plaque-reduction neutralization test (PRNT). Only those animals that did not show evidence of previous dengue exposure were included in the study.

- **Vaccine DNA.** The vaccine construct D1ME contained the dengue-1 pre-membrane (prM) and full-length envelope (E) genes cloned into vector pVR1012 (Vical Inc.) (Raviprakash et al., 2000). The dengue-1 sequence (strain Western Pacific 74) from nucleotide 369 to 2392 (Mason et al., 1987) was amplified by RT–PCR by using the primers 5′ ACATCGGAAGGGATC TTATTTTTGGCTAT and 5′ AGTTGATCCCATTTACGCTGGGACCATGACTCCTTAG. The PCR product was digested with restriction endonucleases HindIII and BamH I and cloned into pVR1012 that was cut with ScaI and BamHI. The vaccine construct was purified by alkaline lysis followed by two isopycnic centrifugations through CsCl gradients and by extensive dialysis with PBS (Maniatis et al., 1984). The purified DNA was tested for expression of dengue-1 E by indirect immunofluorescence of transfected 293 cells (ATCC) with dengue-1 hyperimmune mouse ascitic fluid. pVR1012 vector plasmid DNA and pUC19 DNA were purified by similar procedures. pUC19 DNA was used as a source of immuno-stimulatory sequences (ISS) (Porter et al., 1998).

- **Immunizations.** Monkeys were immunized intramuscularly (i.m.) or intradermally (i.d.) as shown in Table 1. For i.m. vaccinations, 0·5 ml vaccine DNA in PBS was divided into two inoculations of 0·25 ml in each tibialis anterior muscle. For i.d. inoculations, 0·5 ml total volume of vaccine DNA was distributed among six sites in the anterior thoracic area. The vaccinations were repeated at months 1 and 5 after the initial inoculation. The i.d.-vaccinated monkeys (groups 4 and 5) received a fourth inoculation at month 12.

- **Antibody analyses.** The monkeys were bled at monthly intervals and their sera were tested for dengue-1-specific antibody by ELISA and/or PRNT. IgG antibody was detected by ELISA as described previously (Ansari et al., 1993) except that PEG-precipitated dengue-1 virions were used as the antigen and a peroxidase-labelled anti-human Ig was used as the conjugate. Antibody avidity measurements were performed by comparing antibody binding to antigen in the presence and absence of 6 M urea in a standard ELISA (Narita et al., 1998). Briefly, sera were diluted so as to obtain a standard ELISA absorbance of between 0·8 and 1·5. ELISA was performed in duplicate microtitre plates. After antibody binding, both plates were washed three times with wash buffer. To one plate, 200 μl wash buffer was added per well, and 200 μl wash buffer per well containing 6 M urea was added to the other plate. Both plates were incubated for 5 min at room temperature. The buffers were removed and the plates were washed three more times with wash buffer. Incubations with conjugate and substrate were carried out according to

### Table 1. Study design of dengue-1 DNA vaccine trial in rhesus macaques

<table>
<thead>
<tr>
<th>Group</th>
<th>Route</th>
<th>n</th>
<th>D1ME* (1 mg)</th>
<th>pUC19 (0·5 mg)</th>
<th>Vector plasmid (1 mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>i.m.</td>
<td>4</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>i.m.</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>3</td>
<td>i.m.</td>
<td>1†</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>i.d.</td>
<td>2</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>5</td>
<td>i.d.</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>6</td>
<td>i.d.</td>
<td>2</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>None‡</td>
<td>2</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* Vaccine candidate 1012D1ME (dengue-1 prM and E genes cloned into vector pVR1012).
† One of two animals died at month 3 due to unrelated causes.
‡ Dengue-naive animals.

![Fig. 1. Antibody response to i.m. (a) and i.d. (b) immunization of rhesus macaques with dengue-1 DNA vaccine. The IgG antibody levels were measured by an indirect ELISA at 1:100 dilution of the serum samples. Arrows indicate booster doses. The solid bars show vaccine + pUC19; hatched bars, vaccine; open bars, control plasmid.](image-url)
the standard ELISA protocol. The avidity index (AI) was calculated as $100 \times (A_{\text{total}} - A_{\text{free}})/A_{\text{total}}$. An antibody-capture ELISA was used for IgM antibody detection as described previously (Simmons et al., 1998). PRNT was performed with LLC-MK2 cells as described previously (Russell et al., 1967) using twofold serial dilutions of serum samples. A pool of the sera of animals collected before they were primed was used as the negative control for PRNT. Fifty % PRNT titres (PRNT50) were determined by probit analysis. PRNT50 titres were considered to be zero if a 1:10 serum dilution caused less than 50% reduction.

In vitro stimulation of T cells. Peripheral blood lymphocytes (PBL) were purified by Ficoll–Hypaque centrifugation from heparinized blood collected at months 8 and 11 after the initial inoculation. Cells ($3 \times 10^9$) were plated into each well of a 24-well plate and cultured for 4 days in a total volume of 1 ml in the presence of concanavalin A (10 mg/ml), dengue-1 virus ($5 \times 10^8$ p.f.u. or inactivated dengue-1 virus ($5 \times 10^3$ p.f.u. equivalent). Supernatant fluids were assayed for secreted γ- interferon (IFN-γ) by ELISA. Briefly, flat-bottomed microtitre plates (Maxisorp, NUNC) were coated with 100 µl per well mononuclear antibody BMS-107 (anti-human IFN-γ, Alexis Corp.) at 2 µg/ml. The plates were washed once with PBS/0.05% Tween 20 (wash buffer) and blocked for 1 h at 37°C with wash buffer containing 0.5% BSA. Plates were washed five times with wash buffer and 50 µl test samples or IFN-γ standard solution (Endogen) was added to wells and incubated for 2 h at 37°C. Plates were washed as before and 100 µl of biotinylated anti-human IFN-γ (γ interferon (IFN-γ), Diapharma) was added to each well and incubated for 1 h at 37°C. Plates were washed as before and developed by using streptavidin–alkaline phosphatase (100 µl, 1 h, 37°C) and substrate p-nitrophenyl phosphate. The difference in absorbance at 405 and 620 nm was used as the end point. Plates were read every 30 min until the A190 minus A90 for the 1000 pg/ml standard registered a reading between 1:5 and 2:0. The concentration of IFN-γ in the test samples was determined from the standard curve.

For inactivation of dengue-1 virus, 9 ml virus stock (109 p.f.u./ml) was mixed with 1 ml 1 M Tris–HCl (pH 9.0) and 1:1 ml 1% β-propiolactone. The mixture was incubated at 37°C for 1 h with intermittent swirling. The inactivated virus was pelleted by centrifugation at 32000 r.p.m. for 2 h in a Beckman SW41 rotor. The pellet was washed once with RPMI-1640 medium containing 10% foetal bovine serum and resuspended in 0.9 ml of the same medium. To confirm inactivation, a 0.1 ml aliquot of this preparation was tested in a plaque assay with Vero cells.

Virus challenge and viraemia. Two separate virus challenge experiments were conducted. Groups 1, 2, 3 and 6 (Table 1) were challenged at 9 months after the initial immunization. The three monkeys from groups 3 and 6 served as the control group for this challenge experiment. Groups 4, 5 and 7 were challenged at 15 months. The naive monkeys in group 7 served as controls for this experiment. Each monkey was bled prior to challenge and then inoculated subcutaneously in the upper left arm with 0.5 ml of a solution containing 1:25 × 105 p.f.u. of dengue-1 virus. The virus used for challenge (dengue-1 Western Pacific 74) was prepared from infected foetal rhesus lung cells and was kindly provided by K. Eckels (Walter Reed Army Institute of Research). Monkeys were bled daily for 10 days and again on days 14 and 29 after challenge and the sera were used to measure viraemia and antibody responses. Viraemia was measured by inoculating 25 cm2 flasks of confluent Vero cells in duplicate with 0.2 ml of a 1:3-diluted serum sample. The inoculated cells were incubated for 10 days at 37°C in a CO2 incubator. The cells were then scraped off the flask, washed with PBS and spotted in duplicate onto immunofluorescence slides. The slides were processed for indirect immunofluorescent staining by using dengue-1 hyperimmune mouse ascitic fluid and FITC-conjugated anti-mouse Ig and examined under a fluorescence microscope with appropriate positive and negative controls.

Results

Dengue-1 DNA vaccine is immunogenic in rhesus monkeys

A dengue virus-specific antibody response was seen as early as 1 month after priming in monkeys that were vaccinated i.m. (Fig. 1a). There was a trend towards an increase in the antibody response following boosts at months 1 and 5. No significant difference was observed in the antibody levels of monkeys that were co-immunized with pUC19 plasmid versus those immunized with vaccine alone. Compared with the i.m.-vaccinated monkeys, the antibody levels of i.d.-vaccinated monkeys were lower and declined more rapidly over time (Fig. 1b).

The ability of sera of immunized monkeys to neutralize dengue-1 virus was tested by PRNT (Table 2). One month after the second boost (month 6), sera from six of eight i.m.-vaccinated monkeys showed PRNT50 titres ranging from about 1:20 to 1:360. The PRNT50 titres, however, declined over time. As with ELISA, co-immunization with pUC19 DNA did not increase the neutralizing antibody levels significantly. Two of the i.m.-vaccinated monkeys (MV4 and MV8) failed to

Table 2. Neutralizing antibody responses in rhesus macaques after immunization with dengue-1 DNA vaccine

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunization</th>
<th>Animal</th>
<th>Time (months)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>i.m. (−pUC)</td>
<td>MV1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV4</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>i.m. (+pUC)</td>
<td>MV5</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV7</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV8</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>i.d. (−pUC)</td>
<td>DC1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DC2</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>i.d. (+pUC)</td>
<td>DV1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DV2</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>i.d. (+pUC)</td>
<td>DV3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DV4</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>i.d. (control)</td>
<td>DC1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DC2</td>
<td>0</td>
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<tr>
<td>7</td>
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<td>N1</td>
<td>NT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N2</td>
<td>NT</td>
</tr>
</tbody>
</table>

Table 2. Neutralizing antibody responses in rhesus macaques after immunization with dengue-1 DNA vaccine

Data are reciprocal PRNT50 titres. NT, Not tested.

* Day 0 of challenge for groups 1, 2, 3 and 6.
† Day 0 of challenge for groups 4, 5 and 7.
**Table 3.** Viraemia in rhesus macaques immunized with dengue-1 DNA vaccine and challenged with live dengue-1 virus

Viraemia was measured by indirect immunofluorescence of Vero cells exposed to serum samples and incubated for 10 days. Presence and absence of virus are indicated by + and −. NT, Not tested.

<table>
<thead>
<tr>
<th>Expt*</th>
<th>Animal</th>
<th>Time post-challenge (days)</th>
<th>Duration of viraemia (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>Vaccinated</td>
<td>MV1</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV2</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV3</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV4</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV5</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV6</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV7</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MV8</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>MC1</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DC1</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DC2</td>
<td>−</td>
</tr>
<tr>
<td>2</td>
<td>Vaccinated</td>
<td>D1</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>D2</td>
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<tr>
<td></td>
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<td>D3</td>
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<td></td>
<td></td>
<td>D4</td>
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<tr>
<td></td>
<td>Naive</td>
<td>N1</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>N2</td>
<td>−</td>
</tr>
</tbody>
</table>

* Monkeys were challenged at month 9 (4 months after third immunization) (expt 1) or at month 15 (4 months after fourth immunization) (expt 2). † P = 0.012 by Kruskal–Wallis test.

Monkeys vaccinated i.m. are protected from live dengue-1 virus challenge

The eight i.m.-vaccinated monkeys (MV1–MV8) and three control vaccinated monkeys (MC1, DC1 and DC2) were challenged at month 9 by subcutaneous inoculation with 1.25 × 10⁴ p.f.u. dengue-1 virus. These monkeys were bled daily for 10 days and the sera were analysed for the presence of infectious virus. Four of the eight i.m.-vaccinated monkeys were protected completely from developing viraemia (Table 3, Expt 1). The other four i.m.-vaccinated monkeys showed viraemia for 2 or 3 days. The i.m.-vaccinated group thus had a median period of viraemia of 1.5 days. The three control vaccinated monkeys showed a median period of viraemia of 4 days. The reduction in viraemia for the naive controls (Table 3, Expt 2).

In a second challenge experiment, the i.d.-vaccinated monkeys (DV1–DV4), along with two naive control monkeys (N1 and N2), were inoculated with dengue virus at 15 months and viraemia was evaluated as described above. No significant reduction in viraemia was observed in the i.d.-vaccinated monkeys, which had a median period of viraemia of 4 days compared to 4.5 days for the naive controls (Table 3, Expt 2).

**Immunological memory in dengue-1 DNA-vaccinated monkeys**

The sera obtained from animals following challenge with dengue-1 virus were examined by ELISA for the kinetics of IgG and IgM antibody production. For i.m.-vaccinated monkeys, IgM responses were similar to controls except that, on day 29, the levels had dropped in vaccinated monkeys compared with control monkeys (Fig. 2). These i.m.-vaccinated monkeys showed a rapid rise in IgG antibody from day 7 that peaked on day 14 (Fig. 2b). The IgG antibody in the control monkeys was only beginning to rise on day 14 and the levels were still modest on day 29. These results indicate that the i.m.-vaccinated monkeys mounted anamnestic antibody responses to virus challenge, whereas the control monkeys
controls.  

IgG antibody for i.m.- (c) and i.d.- (d) vaccinated monkeys was measured by an indirect ELISA at 1:1000 dilution of serum samples. Solid bars, vaccinated; hatched bars, control rhesus macaques. IgM antibody for i.m.- (a) and i.d.- (b) vaccinated monkeys was measured by an antibody-capture ELISA at 1:100 dilution of serum samples. IgG antibody for i.m.- (a) and i.d.- (b) vaccinated monkeys was measured by an indirect ELISA at 1:1000 dilution of the serum samples. Solid bars, vaccinated; hatched bars, controls.

showed primary antibody responses. Similar results were obtained when the IgM and IgG antibodies of i.d.-vaccinated monkeys were compared to those of naive control monkeys (Fig. 2c, d).

**Antibody avidity**

In order to determine whether there was a qualitative difference between the antibodies produced in animals that were completely protected and those produced in animals that were not completely protected, antibody avidity in the presence of 6 M urea was measured by ELISA. Monkeys MV1 and MV2 (completely protected), MV7 and MV8 (truncated viraemia) and DC1 and DC2 (control) were included in this assay. The results showed that, for vaccinated monkeys, the AI remained constant for sera collected at 7 months and on the day of challenge for the two protected monkeys (MV1, MV2) or increased for the two vaccinated monkeys that developed truncated viraemia (MV7, MV8) (Table 4). One of the two monkeys that was not completely protected (MV8) had an AI comparable to those of protected monkeys. On virus challenge, the control monkeys produced antibody that matured from a very low AI (1 month) to a higher AI (3 months).

**T cell stimulation**

PBL from two naive monkeys and two vaccinated monkeys were stimulated in vitro and the secretion of IFN-γ was measured by ELISA (Table 5). PBL from naive monkeys could not be stimulated by dengue virus. In the two vaccinated monkeys that were tested, PBL from month 11 (2 months after dengue-1 virus challenge) were stimulated, but those from month 8 (3 months after the last immunization but 1 month before challenge) were not. PBL from month 11 were stimulated by both live and inactivated dengue-I virus.

**Discussion**

We have demonstrated that a dengue virus type 1 DNA vaccine elicited neutralizing antibody responses in rhesus macaques. Upon challenge with live dengue-1 virus, half of the i.m.-vaccinated macaques were protected completely from viraemia. The remainder exhibited reduced viraemia compared with the control macaques.

We have shown previously that plasmid DNAs expressing dengue virus type 2 prM and truncated E proteins are immunogenic in mice (Kochel et al., 1997; Porter et al., 1998). We have tested a series of dengue-1 DNA vaccine candidates expressing truncated or full-length E with or without prM and found that the construct expressing dengue-1 prM and full-length E produced long-lived neutralizing antibodies in mice (Raviprakash et al., 2000). Mice are not the desirable challenge model for dengue infection, however. The mouse model involves intracranial inoculation of dengue virus, which causes encephalitis, and mice are less susceptible to infection as they become older (> 6 weeks of age). Historically, rhesus macaques have been used for evaluation of dengue vaccines. These non-human primates become viraemic after subcutaneous inoculation of live dengue virus. The data presented here demonstrate that the dengue-I DNA vaccine expressing prM and E can induce protective immune responses in the rhesus macaque model.

In recent years, the relatively new technology of DNA vaccination for several other pathogens has been tested for immunogenicity and/or efficacy in non-human primates. Wang et al. (1993) first demonstrated seroconversion of cynomolgus macaques upon i.m. inoculation with a plasmid DNA construct expressing the human immunodeficiency virus type 1 (HIV-1) envelope gene. Since then, several different HIV/simian immunodeficiency virus DNA vaccines have been shown to be immunogenic in rhesus macaques (Lekutis et al., 1997; Yasutomi et al., 1996) and chimpanzees (Bagarazzi et al., 1998;
Table 4. Avidity of antibody from rhesus macaques after vaccination with dengue-1 DNA vaccine and virus challenge

Antibody avidity was measured by ELISA in the presence of 6 M urea. The AI was calculated as $100 \times \frac{A_{+ \text{urea}}}{A_{- \text{urea}}}$. ND, Not done.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Vaccine</th>
<th>Protection status</th>
<th>AI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>7 months</td>
</tr>
<tr>
<td>MV1</td>
<td>+</td>
<td>Fully protected</td>
<td>47·1</td>
</tr>
<tr>
<td>MV2</td>
<td>+</td>
<td>Fully protected</td>
<td>55·7</td>
</tr>
<tr>
<td>MV7</td>
<td>+</td>
<td>Reduced viraemia</td>
<td>15·0</td>
</tr>
<tr>
<td>MV8</td>
<td>+</td>
<td>Reduced viraemia</td>
<td>40·5</td>
</tr>
<tr>
<td>DC1</td>
<td>–</td>
<td>Naive control (unprotected)</td>
<td>ND</td>
</tr>
<tr>
<td>DC2</td>
<td>–</td>
<td>Naive control (unprotected)</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Day of challenge.

Table 5. *In vitro* stimulation of PBL of rhesus macaques vaccinated with dengue-1 DNA vaccine and challenged with live dengue-1 virus

Stimulation was monitored by measuring levels of IFN-γ, which are given in pg/ml. Cells were cultured for 4 days in the presence of the stimulant and secreted IFN-γ was measured by ELISA.

<table>
<thead>
<tr>
<th>Stimulant</th>
<th>Vaccinated Before challenge (month 8)</th>
<th>After challenge (month 11)</th>
<th>Control* (unvaccinated, unchallenged)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MV1</td>
<td>MV5</td>
<td>MV1</td>
</tr>
<tr>
<td>Medium only</td>
<td>0</td>
<td>0</td>
<td>28</td>
</tr>
<tr>
<td>Concanavalin A</td>
<td>924</td>
<td>872</td>
<td>912</td>
</tr>
<tr>
<td>Dengue-1 virus</td>
<td>0</td>
<td>0</td>
<td>724</td>
</tr>
<tr>
<td>Inactivated dengue-1</td>
<td>0</td>
<td>0</td>
<td>324</td>
</tr>
</tbody>
</table>

* Control animals (C1 and C2) are dengue-naive monkeys used as controls for this assay. These monkeys were not part of the vaccine study.

Boyer et al., 1997). It was further demonstrated that chimpanzees vaccinated with an HIV-1 DNA vaccine were protected from a high-dose heterologous HIV-1 challenge (Boyer et al., 1997). Similarly, inoculation of cynomolgus macaques with a DNA vaccine for rabies virus elicited protective immune responses comparable to those elicited by the human diploid cell vaccine (Lodmell et al., 1998). Immunogenicity of influenza virus (Donnelly et al., 1995) and hepatitis B virus (Davis et al., 1996; Gramzinski et al., 1998) DNA vaccines in non-human primates have also been reported.

Various routes and doses of DNA vaccines have been used in primate models. Eight μg of a rabies DNA vaccine was effective in cynomolgus monkeys when delivered by gene gun (Lodmell et al., 1998). When delivered i.d. by needle injection, the same dose failed to elicit an immune response; the monkeys seroconverted only after an additional i.m. boost of 250 μg DNA. A Plasmodium DNA vaccine expressing the CSP gene showed a clear dose response when *Aotus* monkeys were immunized i.d. with 2, 0·5 and 0·125 mg doses of DNA (Gramzinski et al., 1997). Immunizations i.m. with similar doses were ineffective. We have delivered 1 mg doses of dengue-1 vaccine DNA by needle injection via the i.m. or the i.d. route. The antibody response was better in i.m.-vaccinated monkeys compared with those that were vaccinated i.d. This could be partly due to the anatomy of the skin of rhesus macaques and the difficulty of achieving ‘true’ i.d. inoculations. In contrast,
we have observed in a separate study that the route of inoculation (i.m. or i.d.) did not affect antibody levels significantly in vaccinated *Aotus* monkeys (Kochel et al., 2000).

DNA sequences containing unmethylated CpG motifs are known to stimulate humoral and cellular immune responses and have been termed ISS motifs (Krieg, 1999). The pUC19 plasmid contains four such ISS motifs and co-immunization of pUC19 with a dengue-2 DNA vaccine candidate resulted in increased antibody responses in mice (Porter et al., 1998). In the present study, no such stimulation was apparent in monkeys that were co-administered pUC19 DNA. It has been shown recently that the optimal ISS motifs for human cells are distinct from the mouse ISS motifs (Hartmann et al., 1999), probably explaining the lack of effect seen in this study of pUC19 co-immunization in non-human primates, which are related more closely to humans than to mice.

It is believed that antibody can protect against dengue virus infection and that neutralizing antibody is an important component of this protection. Maternal transfer of antibody to newborns by dengue-immune mothers is known to protect infants from dengue virus infection and the duration of protection was found to correlate with the presence of neutralizing antibody (Kliks et al., 1988). Also, mice can be protected from dengue virus challenge by the passive administration of monoclonal neutralizing antibody. Our data show a good correlation between protection and neutralizing antibody titres measured 1 month prior to challenge. The four monkeys that had measurable neutralizing antibody at month 8 are the same four that were protected completely from viraemia. At the time of challenge (month 9), although only one monkey had detectable neutralizing antibody, we presume that some biologically active neutralizing antibody below the detection threshold persisted in these animals.

While existing evidence strongly suggests that neutralizing antibody alone can be protective, other immune responses like non-neutralizing antibody or cellular immunity could also be important. For example, in studies involving dengue-2 live-attenuated vaccine (Scott et al., 1980) as well as purified inactivated dengue-2 virus vaccine (Putnak et al., 1996) in rhesus monkeys, viraemia was observed in some monkeys despite neutralizing antibody titres of 1:60 or greater on the day of challenge. Similarly, monkeys that had no detectable neutralizing antibody were protected from developing viraemia (Scott et al., 1980). Mice immunized with recombinant vaccinia virus expressing dengue-4 structural proteins were protected from virus challenge although no detectable antibody could be demonstrated (Bray et al., 1989). Passive transfer of non-neutralizing antibody directed against the non-structural protein NS-1 of dengue virus has been shown to afford partial protection in mice (Falguet et al., 1990). Our study indicates either that very low levels of neutralizing antibody (< 10) are sufficient for protection or that other immune responses such as non-neutralizing antibody or cell-mediated immunity contribute to protection from viraemia. These observations underscore the importance of virus challenge as an end-point in dengue vaccine studies.

The antibody affinity to virus as measured by ELISA did not show marked differences between monkeys that were protected and unprotected. Two of the monkeys, MV7 and MV8, showed an increase in AI from month 7 to month 9, when the PRNT_{50} titres were actually declining. Because of these observations and the small number of samples in our study, no clear conclusion could be drawn about the relationships among the AI, virus neutralization and protection. However, DNA vaccination induced antibodies with AI similar to those induced by virus infection.

All the vaccinated monkeys, i.m. as well as i.d., showed anamnestic antibody responses upon virus challenge, indicating successful priming by the DNA vaccine. An anamnestic antibody response has been shown to be critical for protection of mice from encephalitis when challenged with JE virus following vaccination with a JE virus DNA vaccine (Konishi et al., 1999). An anamnestic antibody response may be important, but not necessarily sufficient, for protection from dengue infection, since the i.d.-vaccinated monkeys were not protected despite mounting such a response. The anamnestic antibody response also suggested that there was virus replication in the vaccinated monkeys, even in the absence of detectable viraemia. It has been shown recently in the case of a tick-borne encephalitis (TBE) virus DNA vaccine that mice vaccinated and challenged with TBE virus did not develop viraemia but did show evidence of virus replication (Kreil et al., 1998). The strong IgM antibody response following challenge of vaccinated monkeys in our study may reflect a primary antibody response against non-structural proteins that are not expressed by the DNA vaccine.

Two representative monkeys (MV1 and MV5) were tested for T-cell memory by *in vitro* stimulation of PBL. Dengue-1 virus could stimulate the T cells only from PBL obtained after challenge. It has been observed that PBLs from monkeys previously infected with dengue virus are more permissive to dengue infection *in vitro* compared with PBLs from dengue-naive monkeys (Marchette & Halstead, 1974). Positive stimulation by inactivated dengue-1 virus indicated that the difference in stimulation of T cells observed for PBL obtained before and after dengue virus challenge is probably not due to a difference in infectability of these cells. The T-cell memory after virus challenge is probably greater because most of the known T-cell epitopes reside in the non-structural proteins (Mathew et al., 1998; Rothman & Ennis, 1999), which were not part of the vaccine construct used in this study. However, the vaccine probably induces T-cell memory, as shown by the anamnestic antibody response following virus infection. Studies utilizing enriched populations of antigen-specific murine memory B cells and T-helper cells have shown that memory B cells require lower concentrations of soluble antigen and fewer T-helper cells to proliferate and differentiate compared with virgin B cells (Gray, 1993; Sanders et al., 1987).
Thus, a small number of memory T cells in our vaccinated monkeys could have gone undetected in our ELISA. It is also possible that, 3 months after the last immunization, the majority of memory T cells are in the lymphoid tissue and not in the peripheral blood.

The dengue DNA vaccine described here is the first flavivirus DNA vaccine to be tested in non-human primates. This result is a successful test of concept, demonstrating that a DNA vaccine can provide protection against dengue viraemia. It suggests that further work is required to improve and assess DNA vaccines for their ability to prevent disease due to dengue infection.

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