INTRODUCTION

Dengue is a mosquito-borne viral disease of humans and a significant public health threat in the tropics and sub-tropics, where billions of people are at risk and an estimated 100 million new infections occur each year.1 There are four dengue virus serotypes: dengue-1, dengue-2, dengue-3, and dengue-4. These viruses form an antigenically distinct subgroup within the flavivirus family.2 They are enveloped, RNA viruses, which encode 10 proteins: 3 virus structural proteins: capsid (C), membrane (M) and its precursor pre-membrane (prM), and envelope (E); and 7 nonstructural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.3 Most of the virus-neutralizing epitopes, which are important for immunity, are located on the virion surface on the structural E glycoprotein.4

Currently, there are no licensed vaccines for dengue, although there are several vaccine candidates in pre-clinical and clinical trials. The leading candidates are live-attenuated virus vaccines in Phase 2 clinical trials in the United States and in Thailand.5−8 There are also continuing efforts to develop alternative vaccine candidates in the event of failure of the live vaccine approach. In this category are vaccines based on chimeric viruses (e.g., dengue structural genes in a backbone of nonstructural genes from another flavivirus), vaccines based on viral replicons, and non-replicating protein vaccines such as purified inactivated whole virus and recombinant subunit antigens.9,10 The virus structural antigens prM, E, and an amino-terminal 80% fragment of E produced by expression of various gene expression systems have been used successfully as immunogens in animal models.11,12 Although these subunit protein vaccines elicit potent humoral immune responses, they may not be as effective as live vaccines at stimulating cell-mediated immune responses, which require intracellular antigen processing and presentation and may be important for the clearance of virus-infected cells and for long-term protection. Protein subunit vaccines can also be difficult to produce and purify, which adds to their cost. Some of these potential drawbacks may be overcome by the use of nucleic acid (DNA) vaccines.

The concept of vaccination with nucleic acids that encode antigenic proteins is now more than 10 years old, having been first proposed by Wolff and others.13 In principal, any antigen-encoding sequence, when cloned into a plasmid vector under the control of an appropriate promoter and expressed intracellularly, can serve as an immunogen to elicit effective humoral and cell-mediated immune responses. DNA vaccination has been used experimentally to immunize successfully against several viral disease threats including influenza,14 rabies,15 hepatitis B,16 and human immunodeficiency virus.17 Recent studies demonstrate that DNA vaccines containing the full-length prM and E (prM-E) genes of dengue-1 and dengue-2 viruses are immunogenic for mice,18,19 and that a dengue-1 prM-E DNA vaccine is also immunogenic and partially protective for rhesus and Aotus monkeys.20,21 However, a DNA vaccine containing only 80% of the E gene of dengue-1 was found to be less immunogenic for mice than full-length prM-E.18 Earlier it was demonstrated that co-expression of full-length prM and E genes is necessary for producing a highly immunogenic, secreted form of the E antigen.22,23 The co-expression of immuno-stimulatory sequences, adjuvants, and antigen trafficking sequences along with the antigen-coding sequences is also possible with DNA vaccines, and some of these approaches have been used in dengue DNA vaccines with some degree of success.24,25

In the present work, a dengue-2 DNA vaccine expressing the prM and E genes was produced and evaluated. The vac-
cine, administered by a gene gun, induced anti-dengue immune responses in mice and rhesus macaques, and the macaques were partially protected against viremia after challenge with live dengue-2 virus. This is the first report of protection of a non-human primate against dengue-2 virus with a DNA vaccine, which adds further support for the utility of dengue DNA vaccines.

MATERIALS AND METHODS

Construction of the vaccine. The vaccine contained the complete prM and E genes obtained from the New Guinea C (NGC) strain of dengue-2 virus, the sequence of which has been previously reported. A template for polymerase chain reaction (PCR)–amplification of the gene region encompassing prM through E was prepared from plasmid pKT2.4, which contained the prM and the N-terminus of E and plasmid pRP2, which contained the C-terminus of E. The plasmids were digested with Eco RI and ligated together at the Eco RI site. The prM-E gene product was amplified by a PCR to exclude the native prM signal peptide and to introduce a stop codon at the end of the full length E gene. The reaction mixture contained 1 ng of template, 0.4 μM of primers GW116 (5′-GAGCTAGCTTCCATTTAACCAC-3′) and GW163 (5′-GGCAGATCTGCTTAGGCTGACCAT-AACTCC-3′), 1.5 mM MgCl₂ (Promega, Madison, WI), 200 μM of each dNTP (U.S. Biochemicals, Inc., Cleveland, OH), 2.5 units of Taq polymerase (Promega), sterile nuclease-free water to give a final volume of 100 μL, and an overlay of mineral oil (Aldrich Chemicals, Inc., Milwaukee, WI). The PCR was performed in a PTC-200 thermocycler (MJ Research, Waltham, MA) that was programmed for the following routine: pre-denaturation for four minutes at 95°C, followed by 30 cycles consisting of one minute at 95°C, 75 seconds at 55°C, and one minute at 72°C. This was followed by 10 minutes at 72°C and cooling to 4°C. Following purification, the insert was digested with Nhe I and Bgl II to create cohesive ends for cloning into the expression vector WRG7063. This vector uses the strong immediate-early promoter and exon1-intron A of the cytomegalovirus, the human tissue plasminogen activator signal peptide to enable extracellular secretion of prM and E proteins, and the bovine growth hormone gene polyadenylation signal. The vector was prepared by removing the hepatitis B core antigen gene from plasmid WRG7063 by digestion with Nhe I and Bgl II. The vector and insert were then ligated together, resulting in plasmid PJV7247 (Figure 1).

Gene expression and antigen detection. To demonstrate dengue virus gene expression, Lipofectin® reagent (Life Technologies, Rockville, MD) was used to transfect plasmid PJV7247 into murine melanoma (B16) cells (American Type Culture Collection [ATCC], Manassas, VA). At 16 hours post-transfection, the cells were metabolically labeled with [35S]-methionine (50 mCi/mL) (ICN, Irvine, CA) in methionine-deficient RPMI 1640 medium (Life Technologies, Rockville, MD) containing 5% dialyzed fetal calf serum (FCS) for three hours, then chased for one hour with R10 medium (RPMI 1640 plus 10% FCS) (Life Technologies). Transfected cell culture supernatant and cell lysate, and mock-transfected cell lysate (control), were harvested separately and immunoprecipitated using anti-dengue-2 mouse hyperimmune ascitic fluid VR222 (ATCC, Rockville, MD) and Zysorbin G (Zymed, San Francisco, CA) according to manufacturer’s instructions. The immunoprecipitated samples were resuspended in sodium dodecyl sulfate–polycrylicamide gel electrophoresis (SDS-PAGE) sample buffer containing 1% β-mercaptoethanol, and subjected to electrophoresis on 4–20% SDS-PAGE gels (Novex, Carlsbad, CA). Pre-stained molecular size markers from 19.8 to 104 kDa (BioRad, Hercules, CA) were run in an adjacent lane for estimating molecular weights. The gels were fixed, processed in Amplify® Fluorographic Reagent (Amersham, Piscataway, NJ), dried onto paper, and autoradiographed to visualize the labeled proteins.

Preparation of cartridges for genetic immunization. For each plasmid to be tested, 25 mg of two-micron gold powder was weighed into a microcentrifuge tube. One hundred microliters of 50 mM spermidine (Aldrich Chemicals, Inc.) was added to the tube and the gold was resuspended by vortexing and brief sonication. Twenty-five micrograms of PJV7247 plasmid DNA was added to the tube, followed by the addition of 100 μL of 10% CaCl₂ (Fujisawa USA, Inc., Deerfield, IL) while gently vortexing to effect precipitation of the DNA onto the gold beads. The precipitation reaction was allowed to proceed for 10 minutes on the bench top, after which the gold beads were collected by brief microcentrifugation and washed three times with absolute ethanol (Spectrum Quality Products, Inc., Gardena, CA) to remove excess precipitation reagents. The washed gold-DNA complex was then resuspended in a solution of 0.05 mg/ml of polyvinylpyrrolidone (PVP) (360 kD; Spectrum Quality Products, Inc.) in absolute ethanol to a volume of 3.6 mL (for mice) or 7.2 mL (for rhesus macaques) administered by a gene gun.
Dengue virus DNA vaccination of rhesus macaques. This slurry was injected into a Tefzel® tube (McMaster-Carr, Chicago, IL) that was positioned in a tube turner (PowderJect Vaccines, Inc., Madison, WI) to coat the inside of the Tefzel® tube with the gold-DNA complex. After the tube turning procedure was completed and the ethanol was dried off, the tubes were cut into 0.5-inch shots of vaccine, which were stored at 4°C in the presence of a desiccator. Each shot contained 0.25 μg of DNA (a function of the amount of gold per shot and the DNA:gold ratio), a parameter that was established previously to be nearly optimal for genetic immunizations (Fuller J, unpublished data). At least one hour before use, the shots were moved to room temperature and loaded into the XR1 gene gun device (PowderJect Vaccines, Inc.) for delivery.

Animals. Work with animals was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals, and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. All procedures were reviewed and approved by the Institute’s Animal Care and Use Committee, and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. For immunization of mice, BALB/c or Swiss Webster mice (4–6 weeks old) were anesthetized with a mixture of Ketaset® (Fort Dodge, Overland Park, KS) and Rompun® (Bayer, Shawnee Mission, KS). The abdominal region of the animals was shaved with electric clippers. Two non-overlapping shots of vaccine (0.25 μg of DNA per shot) were delivered to the shaved area using the XR1 device and 450 pounds/inch² (psi) of USP grade helium. For antibody assays, Swiss Webster mice were vaccinated and then bled six weeks later. For cytotoxic T lymphocyte (CTL) assays, BALB/c mice were vaccinated, given one booster inoculation with the same amount of DNA four weeks later, killed two weeks later, and their spleens were removed. For immunization of non-human primates, healthy, flavivirus non-immune (previously tested negative for antibodies by neutralization assay against all four dengue virus serotypes, and by hemagglutination-inhibition assay against yellow fever, Japanese encephalitis, West Nile, and St. Louis encephalitis flaviviruses), Indian rhesus macaques (males and females, weight = 4.5−13.7 kg) were anesthetized with Ketamine (10 mg/kg) and the abdominal and groin areas were shaved with electric clippers and then cleaned and disinfected with 70% ethanol. A total of 4−8 non-overlapping shots of vaccine (0.25 μg of DNA per shot) were delivered to the shaved area using the XR1 device and 450 pounds/inch² (psi) of USP grade helium. For antibody assays, Swiss Webster mice were vaccinated and then bled six weeks later. For cytotoxic T lymphocyte (CTL) assays, BALB/c mice were vaccinated, given one booster inoculation with the same amount of DNA four weeks later, killed two weeks later, and their spleens were removed. For infection of non-human primates, healthy, flavivirus non-immune (previously tested negative for antibodies by neutralization assay against all four dengue virus serotypes, and by hemagglutination-inhibition assay against yellow fever, Japanese encephalitis, West Nile, and St. Louis encephalitis flaviviruses), Indian rhesus macaques (males and females, weight = 4.5−13.7 kg) were anesthetized with Ketamine (10 mg/kg) and the abdominal and groin areas were shaved with electric clippers and then cleaned and disinfected with 70% ethanol. A total of 4−8 non-overlapping shots of vaccine (0.25 μg of DNA per shot) were delivered to the shaved area using the XR1 device and 450 pounds/inch² (psi) of USP grade helium. Successful inoculations, mild to moderate erythema appeared at the site of inoculation within 20−30 seconds. The purpose of the spinner was to control the distribution of DNA at the inoculation site (Fuller J, unpublished data). When the spinner was used, slightly higher pressures were required to obtain a similar degree of erythema. Following inoculation, the animals were returned to their cages. Booster inoculations were administered at the indicated time intervals (see Results). The animals were bled throughout the study and their sera were assayed for neutralizing antibodies to dengue-2 virus. At one month or at seven months after the last vaccine dose, the vaccinated animals and concurrent non-vaccinated controls were challenged with live dengue-2 virus.

Dengue virus neutralizing antibody assay. Neutralizing antibodies to dengue-2 virus were measured using a plaque-reduction neutralization assay with a 50% plaque-reduction endpoint (PRNT50), as previously described with minor modifications.10

Dengue virus challenge and assay for viremia. Vaccinated and control rhesus macaques were challenged identically by subcutaneous injection with 10,000 plaque-forming units of dengue-2 virus strain S16803. Sera were collected daily after virus challenge for 12 consecutive days. Virus was detected by incubating the sera on Vero cell monolayers for 14 days (with refeeding of the cultures at day 7), after which the 14-day supernatant fluids were harvested and virus was detected by a plaque assay on Vero cells.10

Stable transformation of murine (P815) cells for expressing dengue-2 virus prM-E. Murine P815 cells (TIB 64; ATCC) were maintained in R10 medium. For selection of stably transformed cells, G418 (Calbiochem, San Diego, CA) was added to the medium at a concentration of 20 μg/mL. The plasmid vector used for stable transformation of P815 cells was prepared by removing the dengue-2 prM-E genes insert from plasmid PVJ7247 by digestion with Nde I and Bgl II and cloning it into pcDNA3 (Invitrogen, Carlsbad, CA). The resulting plasmid, pWRG7253, was formulated into cartridges as described earlier in this report, except that PVP was omitted from the ethanol slurry. Approximately two million P815 cells were spread over a 0.25-cm² area on the bottom of a 3-cm tissue culture dish. The cells were inoculated by gene gun at a helium pressure of 200 psi with the pWRG7253 cartridges. Cell maintenance medium was added to the dish and the cells were incubated for two days to allow for recovery and DNA incorporation. The cells were then harvested, washed, resuspended in selective medium, and plated at a concentration of 10 cells/well in 96-well tissue culture plates. The clones that arose after incubation in selective medium were expanded and tested for expression of prM and E antigens as described earlier in this report. An antigen-positive clone (pME/P815) was selected for use as a stimulator and target cell for CTL assays.

Dengue virus CTL assay. The pME/P815 target cells used in the assay were prepared as described earlier in this report. These cells are derived from DBA/2 mice, which share major histocompatibility complex (MHC) determinants with BALB/c mice, the source of the effector cells, and are therefore suitable for use in the assay. The pME/P815 cells served as both stimulators and target cells for this assay. They provided a source of soluble and MHC-presented antigen for the stimulation, and MHC1-presented antigen for the cytolysis. To generate effector CTLs, splenocytes from immunized BALB/c mice were cultured with mitomycin C-treated pME-expressing P815 target cells for seven days in complete R10 medium supplemented with 10 units/ml of recombinant rat interleukin-2 (Collaborative, Bedford, MA). These conditions were previously demonstrated to be optimal for maximizing stimulation without increasing non-specific background lysis. Cytolytic activity was measured on day 7 with a standard chromium-release assay in 96-well U-bottom plates containing 3 × 10⁴ target cells per well. The pME/P815 target cells were labeled with 100 μCi of sodium chromate (⁵¹Cr) (New England Nuclear Life Sciences, Boston, MA) for 30 minutes and washed three times to remove unincorporated chromium. Serial dilutions of effector cells were then added to each well.
Construction of a dengue-2 virus prM-E DNA vaccine and expression in cell culture. The prM and E genes from the New Guinea C strain of dengue-2 virus were cloned into plasmid WRG7063 to make the vaccine plasmid PJV7247 (Figure 1). To demonstrate correct expression of the dengue genes, plasmid PJV7247 was transfected into murine B16 cells that were metabolically labeled with $^{35}$S-methionine. After labeling, the cell culture supernatant fluid and the cell lysate were harvested separately, immunoprecipitated, and subjected to electrophoresis on an SDS-polyacrylamide gel. As shown in Figure 2, E protein was detected in both the culture supernatant and the cell lysate, and prM protein was detected mainly in the lysate.

Dengue-2 virus neutralizing antibody and CTL responses in vaccinated mice. The DNA vaccine was tested in mice for its ability to elicit dengue-2 virus neutralizing antibodies and CTL responses. To assess the humoral immune response to vaccination, 10 outbred Swiss Webster mice were vaccinated with 0.5 µg of DNA. The vaccinated mice along with non-vaccinated controls were bled six weeks later. Serum pools were prepared from two mice each and assayed for dengue-2 virus neutralizing antibodies. As shown in Table 1, anti-dengue-2 reciprocal PRNT$_{50}$ titers from 50 to 380 (geometric mean titer = 180) were observed. To assess the ability of the vaccine to elicit CTL responses, BALB/c mice (n = 3) were vaccinated with 0.5 µg of DNA and given booster inoculations with the same amount of DNA four weeks later. The vaccinated mice, along with one non-vaccinated control, were killed six weeks later and their spleens were harvested. Spleen cells prepared from individual animals were antigen-stimulated for seven days in culture with murine P815 cells expressing prM and E (prME/P815 cells) and cultured with the same $^{51}$Cr-loaded target cells at various E:T ratios. As shown in Table 2, effector-mediated cytolysis of target cells increased from an average of 4.8-fold above background at an E:T ratio of 16:6:1 to an average of 7.8-fold above background at a E:T ratio of 50:1. These data demonstrate that the DNA vaccine was effective at stimulating both humoral and cell-mediated immune responses in mice.

**Figure 2.** Expression of dengue virus genes in plasmid PJV7247. Murine melanoma (B16) cells in culture were transfected with plasmid PJV7247. The cells were labeled with $^{35}$S-methionine 16 hours post-transfection. After labeling, the cell culture supernatant fluid and cells were harvested separately. A cell lysate was prepared. The cell lysate and supernatant fluid were immunoprecipitated with anti-dengue-2 hyperimmune mouse ascitic fluid and the immunoprecipitates were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The separated proteins were visualized by fluorography. Lane 1, molecular mass markers (apparent molecular weights indicated in kilodaltons (KD) to the left); lane 2, transfected cell culture supernatant fluid; lane 3, transfected cell lysate; lane 4, transfected cell culture supernatant fluid concentrated 2×; lane 5, lysate from mock-transfected cells. E = envelope; prM = premembrane.

**Table 1**

<table>
<thead>
<tr>
<th>Mouse serum pools</th>
<th>Neutralizing antibody (PRNT$_{50}$) titers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool 1 (vaccinated)</td>
<td>380</td>
</tr>
<tr>
<td>Pool 2 (vaccinated)</td>
<td>130</td>
</tr>
<tr>
<td>Pool 3 (vaccinated)</td>
<td>230</td>
</tr>
<tr>
<td>Pool 4 (vaccinated)</td>
<td>50</td>
</tr>
<tr>
<td>Pool 5 (vaccinated)</td>
<td>360</td>
</tr>
<tr>
<td>Pool 6 (control)</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* Results shown are reciprocal 50% plaque-reduction neutralization (PRNT$_{50}$) titers obtained by virus plaque-reduction neutralization assay using the indicated serum pools and dengue-2 strain X1603 virus.
failed to increase following two additional doses. One month after the fourth dose, the vaccinated animals and non-vaccinated controls were challenged with live dengue-2 virus. Sera obtained for 12 consecutive days after challenge were assayed for the presence of the challenge virus (viremia). Two of three vaccinated animals had no viremia after challenge, while the remaining vaccinated animal had one day of viremia. The three non-vaccinated controls had 4, 4, and 6 days of viremia, respectively.

A second experiment was performed in rhesus macaques to evaluate different vaccination regimens using less DNA per dose and fewer doses. In this experiment, three dengue-naive animals were vaccinated with 1 \( \mu \)g of DNA and given booster inoculations two months later with 1 \( \mu \)g of DNA (Table 4, group A), three animals were vaccinated with 1 \( \mu \)g of DNA but not given booster inoculations (group B), and three animals served as concurrent, non-vaccinated controls (group C). The results in Table 4 show that one of three animals in group A that received two 1-\( \mu \)g doses made neutralizing antibody (A-2). None of the animals in group B that received a single 1-\( \mu \)g dose made neutralizing antibody. All of the animals in groups A, B, and C were challenged one month after vaccination. Following challenge, two of three animals in group A had no viremia (A-1 and A-2) and the remaining animal had one day of viremia (A-3). All three animals in group B had viremia for two, three, and five days, respectively. The non-vaccinated controls (group C) each had four days of viremia.

### Table 2

<table>
<thead>
<tr>
<th>Source of spleen cells</th>
<th>Effector to target (E:T) ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50:1</td>
</tr>
<tr>
<td>1 (vaccinated mouse)</td>
<td>24%</td>
</tr>
<tr>
<td>2 (vaccinated mouse)</td>
<td>29%</td>
</tr>
<tr>
<td>3 (vaccinated mouse)</td>
<td>40%</td>
</tr>
<tr>
<td>Average of all vaccinated mice</td>
<td>31%</td>
</tr>
<tr>
<td>4 (control mouse)</td>
<td>4%</td>
</tr>
</tbody>
</table>

* Results are the percentages of dengue-2 virus premembrane-envelope–expressing target cells that were lysed by effector cells (mouse spleen cells) at the indicated E:T ratios.

A third experiment was performed to determine the level of protection against viremia at a longer interval (i.e., seven months) after vaccination. For this experiment, three dengue-naive rhesus macaques were vaccinated with 1 \( \mu \)g of DNA and given booster inoculations two months later with 1 \( \mu \)g of DNA (Table 4, group D), and three animals served as concurrent, non-vaccinated controls (group E). All of the animals in groups D and E were challenged seven months after vaccination. The results shown in Table 4 demonstrate that one of three vaccinated animals (D-2) made neutralizing antibody and the antibody in this animal persisted to the time of challenge. Following challenge, this animal developed no viremia. The other two vaccinated animals had three and five days of viremia, respectively, and the three non-vaccinated controls (group E) had four, five, and nine days of viremia.

One month after challenge, all vaccinated animals and controls were bled and the sera assayed for neutralizing antibodies to dengue-2 virus. As shown in Tables 3 and 4, neutralizing antibody titers increased in all animals as a result of virus challenge.

### DISCUSSION

With the aim to develop a DNA vaccine for dengue, a plasmid containing the prM and E genes of dengue-2 virus was constructed and formulated onto gold microspheres for delivery by gene gun. The vaccine was immunogenic in mice and non-human primates, and preliminary experiments in rhesus macaques suggest that it protected against viremia after challenge with live dengue-2 virus.

Correct expression of the DNA vaccine was demonstrated by transfection of cultured cells. Both E and prM proteins were found inside the cells, and E protein was found in the extracellular supernatant fluid. This is similar to what is seen in virus-infected cells, where prM and E proteins are found in close association within the infected cell on the surface of immature virions, which then undergo maturation and secretion accompanied by the cleavage of prM to M. It is unclear why the virion-associated M protein was not detected in the extracellular supernatant fluid, but this may be due to its small size (approximately 8 kD), to low levels of antibodies to M protein in the antiserum, or to inefficient labeling.

Expression of the DNA vaccine was then demonstrated in vivo. In mice, the DNA vaccine elicited high-titered neutralizing antibodies to dengue-2 virus and dengue-specific CTL responses, which may be important for protection. However, since results in mice may not be the best predictor of vaccine efficacy in humans, a non-human primate model was used for assessing protection with the DNA vaccine. In this study, rhesus macaques were subjected to a peripheral challenge with live dengue virus, which caused viremia in unvaccinated controls. Although the rhesus macaque is not a disease model, protection against viremia may be a useful marker for predicting the protection of humans against disease.

In the first experiment in rhesus macaques, reciprocal titers of neutralizing antibodies to dengue-2 virus ranging from 40 to 170 were seen following administration of two 2-\( \mu \)g doses of DNA. Interestingly, the titers did not increase after two additional doses and the reason for this failure to increase immunity is not known. When challenged with live dengue-2 virus one month after the fourth dose, two of three vaccinated animals had no detectable viremia and the remaining animal...
had only one day of viremia, compared with an average of 4.7 days of viremia for the controls. The mechanism of protection is unclear since only one of the two protected animals had measurable neutralizing antibodies at the time of challenge. One possibility is that the vaccine generated a protective, dengue-specific CTL response. In fact, specific CTL responses were demonstrated to be important for protection against a related virus, hepatitis C virus.29 Methods for the assessment of CTL responses in rhesus macaques following vaccination and challenge are being developed, and will be attempted in future experiments using frozen peripheral blood mononocytes. Another possibility is that non-neutralizing antibodies, which are not measured by the in vitro neutralization assay, also played some role in protection, e.g., by antibody-dependent cellular cytotoxicity or complement-mediated immune cytolysis of dengue-infected cells, as was demonstrated for antibodies to the yellow fever virus NS1 antigen.30 To distinguish among these possibilities will require additional studies.

Because a six-month, four-dose vaccination schedule with eight inoculation sites per dose is impractical for a human vaccine, a second experiment was performed in rhesus macaques that received two 1-µg doses of DNA made measurable neutralizing antibodies at one month after vaccination with two 1-µg doses of DNA. Following the second dose, one of three animals developed neutralizing antibodies, as was seen in the previous experiment. This animal, which still had detectable neutralizing antibodies at the time of virus challenge, was the only one protected against viremia; neither of the two animals without neutralizing antibody was protected. This suggests that the protective efficacy of two 1-µg doses decreased between one and seven months after vaccination. However, due to the small sample size in these experiments, apparent differences between groups could have been due to chance alone.

In all of the vaccination-challenge experiments reported here, even those animals that demonstrated complete protection against viremia developed a post-challenge neutralizing antibody response. The challenge virus dose of $10^4$ plaque-forming units was probably insufficient by itself for inducing such an antibody response, even in primed animals, without some virus replication. That this (possibly local) replication did not progress to viremia in all vaccinated animals suggests that the DNA vaccine may have elicited an effective anamnestic antibody response, which served to abort further virus replication. This animal, which still had detectable neutralizing antibodies at the time of virus challenge, was the only one protected against viremia; neither of the two animals without neutralizing antibody was protected. This suggests that the protective efficacy of two 1-µg doses decreased between one and seven months after vaccination. However, due to the small sample size in these experiments, apparent differences between groups could have been due to chance alone.

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Although higher doses of DNA may have resulted in better immunogenicity and protection, practical and safety issues limit the amount of DNA that can be administered at one time. Delivery of DNA by particle bombardment using the gene gun is a highly efficient method for achieving immune responses that are equivalent or superior to those achieved using conventional methods, e.g., needle and syringe, but with much lower doses of DNA. Nevertheless, increased dosages may be possible without increasing the number of inocula-

### Table 4

<table>
<thead>
<tr>
<th>Animal*</th>
<th>Vaccine doses</th>
<th>Challenge</th>
<th>Antibody post-dose†</th>
<th>Viremia after challenge‡</th>
<th>Antibody after challenge§</th>
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<tbody>
<tr>
<td>A-1</td>
<td>2</td>
<td>1 month</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>None</td>
</tr>
<tr>
<td>A-2</td>
<td>2</td>
<td>1 month</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>None</td>
</tr>
<tr>
<td>A-3</td>
<td>2</td>
<td>1 month</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>None</td>
</tr>
<tr>
<td>B-1</td>
<td>1</td>
<td>1 month</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>Days 3, 5</td>
</tr>
<tr>
<td>B-2</td>
<td>1</td>
<td>1 month</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>Days 3, 4, 5</td>
</tr>
<tr>
<td>B-3</td>
<td>1</td>
<td>1 month</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>Days 3, 4, 5, 6, 7</td>
</tr>
<tr>
<td>C-1</td>
<td>0</td>
<td>1 month</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>Days 3, 5</td>
</tr>
<tr>
<td>C-2</td>
<td>0</td>
<td>1 month</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>Days 3, 4, 5</td>
</tr>
<tr>
<td>C-3</td>
<td>0</td>
<td>1 month</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>Days 3, 4, 5, 6, 7</td>
</tr>
<tr>
<td>D-1</td>
<td>2</td>
<td>7 months</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>Days 3, 4, 5, 6</td>
</tr>
<tr>
<td>D-2</td>
<td>2</td>
<td>7 months</td>
<td>24</td>
<td>45</td>
<td>None</td>
</tr>
<tr>
<td>D-3</td>
<td>2</td>
<td>7 months</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>Days 4, 5, 7</td>
</tr>
<tr>
<td>E-1</td>
<td>0</td>
<td>7 months</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>Days 3, 4, 5, 6</td>
</tr>
<tr>
<td>E-2</td>
<td>0</td>
<td>7 months</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>Days 3, 4, 5</td>
</tr>
<tr>
<td>E-3</td>
<td>0</td>
<td>7 months</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>Days 1, 2, 3, 4, 5, 6, 7, 8, 9</td>
</tr>
</tbody>
</table>

* The animals in group A received two 1-µg doses, two months apart and were challenged seven months later; animals in group C were unvaccinated and served as controls for groups A and B. Animals in group D received two 1-µg doses, two months apart and were challenged seven months after the second dose; animals in group E were unvaccinated and served as controls for group D.
† Antibody titers (50% plaque-reduction neutralization titer [PRNT₅₀]) were determined by virus plaque-reduction neutralization assay using dengue-2 strain S16803 virus and sera obtained one to two months after each of the four inoculations.
‡ Antibody titers were determined by PRNT₅₀ assay on sera obtained one month after challenge.
§ Antibody titers were determined by PRNT₅₀ assay on sera obtained one month after challenge.

## References

1. Anderson CM, Goldsmith SB, Purcell RH. Antibody post-dose† to dengue-2 virus neutralizing antibodies and protection against virus challenge in rhesus macaques following one to two 1 µg doses of dengue-2 DNA vaccine.

2. The animals in group A received two 1-µg doses, two months apart and were challenged one month after the second dose; animals in group B received on 1-µg dose and were challenged one month later; animals in group C were unvaccinated and served as controls for groups A and B. Animals in group D received two 1-µg doses, two months apart and were challenged seven months after the second dose; animals in group E were unvaccinated and served as controls for group D.

3. Antibody titers (50% plaque-reduction neutralization titer [PRNT₅₀]) were determined by virus plaque-reduction neutralization assay using dengue-2 strain S16803 virus and sera obtained one to two months after each of the four inoculations.

4. Antibody titers were determined by PRNT₅₀ assay on sera obtained one month after challenge.

5. Antibody titers were determined by PRNT₅₀ assay on sera obtained one month after challenge.

6. Antibody titers were determined by PRNT₅₀ assay on sera obtained one month after challenge.

7. Antibody titers were determined by PRNT₅₀ assay on sera obtained one month after challenge.

8. Antibody titers were determined by PRNT₅₀ assay on sera obtained one month after challenge.

9. Antibody titers were determined by PRNT₅₀ assay on sera obtained one month after challenge.

10. Antibody titers were determined by PRNT₅₀ assay on sera obtained one month after challenge.
tions by increasing the gene ratio or the amount of gold per shot, a parameter that is nearly maximal for the delivery of gold particles to the thin skin of rhesus macaques (Fuller J, unpublished data). In a recent clinical trial in which the gene gun was used to administer a hepatitis B DNA vaccine, increasing the amount of DNA per dose resulted in increased rates of seroconversion but had no effect on the geometric mean antibody titers that were achieved following the first or second immunizations.32

Other strategies for increasing the immunogenicity of dengue DNA vaccines have been considered, such as the use of better promoters, the incorporation of promoter-enhancer elements into the vector for higher levels of gene expression, the inclusion of other genes that express potentially protective dengue epitopes, e.g., NS1 and NS3,33,34 and the inclusion of accessory factors such as antigen-trafficking sequences, adjuvants, and immunomodulators. A dengue-2 plasmid DNA vaccine containing lysosomal associated membrane protein trafficking sequences and a granulocyte-macrophage colony-stimulating factor plasmid demonstrated increased immunogenicity in mice.29 Whether these strategies will be effective for humans must still be determined. Our goal is to develop a safe, immunogenic, and effective dengue DNA vaccine for protecting against all four dengue virus serotypes.

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