An Alphavirus Vector-Based Tetravalent Dengue Vaccine Induces a Rapid and Protective Immune Response in Macaques That Differs Qualitatively from Immunity Induced by Live Virus Infection

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Despite many years of research, a dengue vaccine is not available, and the more advanced live attenuated vaccine candidate in clinical trials requires multiple immunizations with long interdose periods and provides low protective efficacy. Here, we report important contributions to the development of a second-generation dengue vaccine. First, we demonstrate that a nonpropagating vaccine vector based on Venezuelan equine encephalitis virus replicon particles (VRP) expressing two configurations of dengue virus E antigen (subviral particles [prME] and soluble E dimers [E85]) successfully immunized and protected macaques against dengue virus, while antivector antibodies did not interfere with a booster immunization. Second, compared to prME-VRP, E85-VRP induced neutralizing antibodies faster, to higher titers, and with improved protective efficacy. Third, this study is the first to map antigenic domains and specificities targeted by vaccination versus natural infection, revealing that, unlike prME-VRP and live virus, E85-VRP induced only serotype-specific antibodies, which predominantly targeted EDIII, suggesting a protective mechanism different from that induced by live virus and possibly live attenuated vaccines. Fourth, a tetravalent E85-VRP dengue vaccine induced a simultaneous and protective response to all 4 serotypes after 2 doses given 6 weeks apart. Balanced responses and protection in macaques provided further support for exploring the immunogenicity and safety of this vaccine candidate in humans.

Dengue fever (DF) is a viral disease characterized by severe headache, skin rash, and debilitating muscle and joint pain. Severe cases are associated with circulatory failure, shock, coma, and death. The disease is caused by any of 4 dengue virus serotypes, 1, 2, 3, and 4 (DENV1 to -4), members of the family *Flaviviridae*, which are transmitted among humans by the mosquito *Aedes aegypti*. DENVs are considered the most important emerging human arboviruses, with worldwide distribution in the tropics, and are responsible for an estimated 36 million cases of DF, more than 2 million cases of severe dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS), and more than 21,000 deaths every year (1–4). Currently, there are no licensed dengue vaccines available.

The development of a dengue vaccine faces unique challenges. The 4 serotypes cocirculate in areas of endemicity, and preexisting immunity to one serotype confers only short-term protection against infection with other serotypes. In addition, epidemiological studies suggest that preexisting cross-reactive antibodies and/or T cells elicited by one serotype may enhance the severity of disease upon infection with a heterologous serotype (reviewed in reference 5). This is partly attributed to the phenomenon of antibody (Ab)-dependent enhancement (ADE), by which preexisting cross-reactive, nonneutralizing antibodies can enhance infection and the disease severity of a subsequent infection (6–8). Therefore, for a dengue vaccine to be safe and effective, it has to induce long-lasting protective immunity to all 4 serotypes simultaneously and must do so soon after immunization to avoid sensitizing the vaccine recipient to enhanced disease. While it is clear that both antibody and T cell immunities play a role in protection from disease and viral clearance, it is not fully understood what qualities

of the immune response, in terms of potency, breadth, and antigen/epitope specificities, are required for a vaccine to be protective in humans.

Most dengue vaccine research efforts for the past 2 decades have been to develop live attenuated virus (LAV) vaccine platforms. A major challenge of this platform has been achieving sufficient attenuation for each monovalent component while preserving immunogenicity for all 4 serotypes. There are three LAV vaccine candidates under active clinical development (9–11). These vaccines are based on recombinant attenuated and chimeric viruses representing each of the four dengue serotypes. In the chimeric yellow fever-dengue tetravalent vaccine, ChimeriVax/CYD-TV, used by Acambis/Sanofi Pasteur, each component contains the genome of the yellow fever vaccine strain 17D, in which the premembrane (prM) and envelope (E) genes of each dengue virus serotype have been substituted for the analogous yellow fever genes (12–14). In the case of the tetravalent vaccine (TetraVax-DV) developed by the Laboratory of Infectious Diseases at the National Institute of Allergy and Infectious Diseases (NIAID), each component has been attenuated either by defined attenuating deletion mutations in the 3' untranslated region (UTR) or by chimerization (15, 16). In the case of the chimeric DENVax devel-

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oped by the U.S. CDC and Inviragen, each component is a chimeric virus containing the prM and E genes of DENV1, -3, and -4 in the genome background of the attenuated vaccine strain DENV2 PDK-53 developed by Mahidol University, Bangkok, Thailand (reviewed in reference 11). These three LAV vaccine candidates are in different stages of clinical phase 1 and 2 trials and have shown short-term safety and immunogenicity (17, 18; J. E. Osorio, Presented at the Third Panamerican Dengue Research Network Meeting, Cartagena, Colombia, 2012). The first efficacy data for a LAV were recently reported from a phase 2b study conducted in Thailand with Sanofi Pasteur's CYD tetravalent vaccine (19). Although the geometric mean neutralizing-Ab levels against the 4 serotypes were higher in vaccinees than in controls, only modest levels of overall protection (~30%) and no protection against DENV2 were observed in the study (19). Although ongoing phase 3 trials may reveal improved efficacy with a larger number of study subjects, these early clinical trial results underscore the need to better understand qualitative and quantitative requirements for a vaccine-induced protective immune response.

Another major challenge faced by LAV vaccine platforms is interference among the four serotype components in the vaccine, causing some serotypes to be dominant over or to interfere with other serotypes in the tetravalent mixture. This has been more evident in the case of the Sanofi Pasteur vaccine and with older LAV attempts no longer in active development (20, 21). This problem was overcome with a three-dose LAV vaccine schedule, administered over a 1-year period in the case of the Sanofi Pasteur vaccine. However, such a long immunization schedule would be difficult to complete in resource-poor countries. Under this scenario, vaccinees would be at increased risk during the 12-month vaccination period, and this increased risk would be long term if an individual received only one or two of the required vaccine doses. While these theoretical concerns are being addressed in clinical trials, there is an urgent need to accelerate the development of second-generation dengue vaccines into clinical trials, with emphasis on overcoming viral interference among the tetravalent vaccine components and utilizing shorter dosing intervals. A number of nonpropagating vaccine strategies are in development. They include (i) adjuvanted recombinant E protein expressed in *Drosophila* S2 cells (reviewed in reference 22), currently in phase 1 clinical testing; (ii) plasmid DNA expressing prM and E proteins, currently in phase 1 clinical testing (23); (iii) adjuvanted inactivated virus (24); (iv) modified adenovirus and alphavirus as viral vectors expressing DENV envelope protein sequences (25–28); and (v) recombinant E domain III (29–32; reviewed in references 33 and 34).

The envelope of DENV contains two transmembrane glycoproteins, envelope (E) and premembrane/membrane (prM/M). E, which is involved in viral binding and entry into cells, is the most important target of neutralizing antibodies (35). The E protein is about 500 amino acids long. The ectodomain (N-terminal 400 residues) has been crystallized, and the atomic structure shows 3 β -barrel domains, represented as EDI, EDII, and EDIII (36). Mouse monoclonal antibodies (MAbs) that neutralize DENV map to all three domains (35). However, the monoclonal antibodies with the strongest neutralization are serotype specific and map to EDIII (37–42), which is a region of ~100 amino acids that folds into an immunoglobulin-like domain and has been implicated in host receptor binding (43). The minor surface glycoprotein, prM/M, plays a role in proper folding of E and particle

assembly and is present as uncleaved prM in the immature particle and partly or fully cleaved M in the mature particle (44).

The antigenic composition and the relative contributions of neutralizing and potentially enhancing epitopes are important considerations in the design of a safe and effective dengue vaccine. However, important knowledge gaps still exist. It is unclear whether mimicking the human immune response to natural infection would be the best vaccination strategy, and the main epitopes on DENV targeted by strongly neutralizing antibodies in the human immune sera remain to be defined. Recent studies have begun to define the specificity of the human antibody response to dengue virus by studying human immune sera and human monoclonal antibodies (reviewed in reference 45). A major population of highly cross-reactive, weakly neutralizing, and infection-enhancing specific antibodies that bind to prM are elicited in response to natural dengue infection (46). The fusion loop in EDII is also a major target of antibodies present in human immune sera (47). Human MAbs with neutralizing activity bind to (i) complex epitopes preserved on the intact virion but not on recombinant E protein (48), (ii) E domains I/II, and (iii) epitopes on the lateral ridge and A strand of EDIII (49). It is important to note that human MAbs with infection-enhancing activity have mapped to prM, EDI/II, and EDIII (50).

Alphavirus-derived replicon vectors have been developed as a vaccine platform (51). Venezuelan equine encephalitis virus (VEE) replicon particles (VRP) are defective, nonpropagating virus-like particles that contain a modified genome expressing high levels of a vaccine antigen. VRP target dendritic cells in the draining lymph nodes of immunized animals (52), where amplification of the replicon RNA results in strong induction of innate immunity and the vaccine antigen is expressed in immunogenic amounts (53, 54). Alphavirus replicon particle vaccines have been studied extensively in recent years and have conferred protective immunity to a wide variety of viral and bacterial antigens tested in different animal models with an exceptional safety record (55–60). More importantly, the layered safety features of the VRP system have been assessed in humans in phase I clinical trials of VRP expressing the Gag protein of clade C HIV (61) and cytomegalovirus gB or a pp65/IE1 fusion protein (62).

The feasibility of the VRP platform as a dengue vaccine was demonstrated previously in mice. Two doses of DENV2 prME-VRP were immunogenic and protective in BALB/c mice (28) and, when formulated as a tetravalent VRP cocktail, induced balanced responses that lasted at least 71 weeks after the second dose (L. J. White, unpublished data). Also, Chen et al. reported that heterologous prime/boost immunizations with DENV1 prME DNA prime/VRP boost were immunogenic and protective in nonhuman primates (NHPs) (63).

The goals of this study were (i) to determine how the magnitude and quality of the antibody responses induced by different vaccine antigen configurations compare to antibody responses induced after dengue virus infection and (ii) to determine whether the VRP vaccine overcomes some of the challenges faced by first-generation LAV dengue vaccines. We first compared in macaques the antibody responses induced by vaccination with monovalent VRP expressing two configurations of E protein, prME and E85, with those induced by infection with live virus. We then constructed and evaluated a tetravalent VRP vaccine expressing the antigen that best performed in the monovalent test, E85. This tetravalent formulation provided 100% seroconversion against all

4 serotypes after only 2 vaccine doses given 6 weeks apart, with no evidence of interference among the components of the vaccine. Protection against virus challenge from each serotype was demonstrated, and antivector immunity against the first dose did not seem to reduce the effectiveness of a second dose. These results suggest that the tetravalent VRP dengue vaccine should receive serious consideration as a next-generation dengue vaccine candidate.

MATERIALS AND METHODS

Cells. BHK-21 and Vero-81 cells were obtained from the American Type Culture Collection (ATCC). The BHK-21 cells were maintained in alpha minimal essential medium (alpha-MEM) containing 10% donor calf serum, 10% tryptone phosphate broth, penicillin (100 U/ml), and streptomycin (100 µg/ml) in the presence of 5% CO₂. Vero-81 cells were maintained at 37°C in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) in the presence of 5% CO₂. Insect C6/36 cells were obtained from the ATCC and maintained at 28°C in alpha minimal essential medium containing 5% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 µg/ml) in the presence of 5% CO₂.

Viruses. Contemporary clinical DENV isolates with low cell culture passage histories were used as the sources of dengue virus genes to be expressed by the VRP vaccine vectors. The sequences are identified by their University of North Carolina (UNC) nomenclature and place and year of isolation: serotype 1, strain UNC 1017, Sri Lanka, 2004; serotype 2, strain UNC 2055, Brazil, 2008; serotype 3, strain UNC 3001 (genotype III), Sri Lanka, 1989; serotype 4, strain UNC 4019, Colombia, 2006.

DENV strains used for monkey challenge studies were obtained from Steve Whitehead (NIAID). DENV1 WP, Western Pacific, 1974 (accession number [AY145121](#)); DENV2 NGC, New Guinea, 1944 (accession number [AY243467](#)); DENV3 Sleman/78, Indonesia, 1978 (accession number [AY648961](#)); and DENV4-814669, Dominica, 1981 (accession number [AF326573](#)) have been used in previous dengue virus challenge studies in monkeys due to their ability to replicate and cause detectable viremia in macaques (64). They were passaged once in Vero cells in our laboratory and stored at -80°C. The viruses used in neutralization assays have been assigned by the World Health Organization as dengue virus reference strains and were provided by R. Putnak from WRAIR (DENV1 WP, DENV2 S-16803, DENV3 CH53489 [genotype II], and DENV4 TVP-360). In addition, we used strains from DENV3 genotype I (UNC 3043, strain 059.AP-2 from the Philippines, 1984), and DENV3 genotype IV (UNC 3036, strain 1342 from Puerto Rico, 1977). These viruses were propagated two or three times in C6/36 cells, titrated on Vero cells, and stored at -80°C. The virus preparations used as antigens to coat enzyme-linked immunosorbent assay (ELISA) plates were obtained by further purification and concentration of the C6/36-grown virus stock, as described previously (65).

Cloning of DENV prME and E85 into VEE vectors. DENV genes were cloned into the VEE replicon plasmid, pVKE (pVR21 with KanR and EcoRV) (28), as follows. Viral RNA was extracted from virus growth culture media and used as the template to generate cDNA by reverse transcription-PCR. The forward PCR primer was designed to insert an EcoRV restriction site at the 5' end, where the last 3' G constitutes the first nucleotide of the first methionine codon, followed by the second and third nucleotides of the Met start codon (AT). The reverse primer was designed to add a stop codon followed by an AscI or PstI site at the 3' end. The sequences of the primers used to amplify the different constructs are as follows: for DENV1 prME, forward, 5'-GGGGTTTAAAAAGAGATCTCAAGC-3', and reverse, 5'-GCCATTAATTAACCTGACCATGACTCC-3'; for DENV1 E85, forward, 5'-GTCCATCACCAGAAAGGATC-3', and reverse, 5'-GCCATTAATTAACCTAGGAACCGAAGTCCCATG-3'; for DENV2 prME, forward, 5'-GGGGTTTCAGGAAAGAGATG-3', and reverse, 5'-GCTTTTAATTAACCTAAGCCTGCACCATAGCTC-3'; for DENV2 E85, forward, 5'-GACACACTTCCAAAGGGCCTTG-

3', and reverse, 5'-GCTATTAATTAACCTAGGATCCAAAATCCCAGGC-3'; for DENV3 prME, forward, 5'-GGGCTTCAAGAAGGAGATCTCAACATGCTGAGCATAATCAAC-3', and reverse, 5'-GGTGCAGGCGCGCCCTAAGCTTGTACCACAGCTCCCAGATAGAGTGTAAAT-3'; for DENV3 E85, forward, 5'-GTCCTTGACCCAGAAAGGTGTTATTTTATACTACTAATGCTGGTCAACCCATCCATGACAAATGAGATGTGTGGGAATAGGA-3', and reverse, 5'-GGTGCAGGCGCGCCCTATGATCCAAAGTCCCAAGCTGTGTCTCCCAAGAT-3'; for DENV4 prME, forward, 5'-GGGATTCAGGAAGGAGATAGGC-3', and reverse, 5'-GCATTAATTAACCTACGCTTGAACCGTGAAGCC-3'; for DENV4 E85, forward, 5'-GACAGGAATCCAGCGAACAGTC-3', and reverse 5'-GCCATTAATTAACCTAGGAACCAAAATC-3'. The prME cassette contained sequences encoding the last 18 residues from the capsid protein serving as a signal peptide for prM, followed by full-length prM and full-length E. The E85 construct contained a C-terminally truncated soluble form of E that represents 85% of the protein, including sequences encoding the E signal peptide (the last 18 residues from prM); domains I, II, and III of the ectodomain; the first alpha helix, H1, and part of the conserved sequence, CS (E residues 1 to 424 for DENV1, -2, and -4 and residues 1 to 422 for DENV3), and lacking the second alpha helix, H2, and the 2 transmembrane domains. The amplified regions were digested with AscI or PstI and cloned into the VEE replicon vector pVKE digested with EcoRV and AscI or PstI.

Assembly and purification of VRP. Production of VRP expressing dengue virus antigens was done as described previously (28). Briefly, the clones were linearized at a unique NotI site downstream of the VEE 3' untranslated region and poly(A) tract, and full-length T7 transcripts were generated *in vitro* using an mMessage mMachine kit (Ambion) as previously described. To package the recombinant replicon genome into VRP for delivery *in vitro* and *in vivo*, the replicon RNA was mixed with two helper RNAs encoding the capsid gene and the attenuated glycoprotein from V3014, also transcribed *in vitro* using T7 polymerase, as previously described (28). The transcripts were cotransfected into BHK cells by electroporation. The culture medium was harvested at 22 to 24 h postelectroporation and clarified, and the VRP were partially purified and concentrated by ultracentrifugation. Each VRP preparation was safety tested as described previously to ensure the absence of propagation-competent virus that could have arisen by nonhomologous recombination during packaging (28). VRP titers were determined in BHK cells by indirect immunofluorescence assay (IFA), using mouse polyclonal antibody against VEE nonstructural proteins, and expressed as IU/ml (28).

***In vitro* expression of DENV E protein in VRP-infected BHK cells.** Subconfluent BHK-21 cells grown in 24-well plates were mock infected or infected at an MOI of 10 with VRP expressing DENV3 prME or E85 in duplicate. At 19 h postinfection, culture medium (0.5 ml) was collected and clarified by centrifugation. The cells were washed with cold phosphate-buffered saline (PBS) and lysed with 100 µl of NP-40 lysis buffer in the presence of protease inhibitors. Samples were analyzed for the presence of E protein by nonreducing SDS-PAGE, followed by Western blotting. Each lane was loaded with 35 µl of cell lysate (out of 100 µl/infected well) or 35 µl of clarified culture medium (out of 0.5 ml/infected well). The electrophoresed proteins were transferred onto polyvinylidene difluoride (PVDF) membranes and probed with mouse MAb 4G2, which binds to EDII of the flavivirus E protein. Following incubation with goat anti-mouse IgG-horseradish peroxidase (HRP), the membranes were developed using enhanced chemiluminescence (ECL) substrate.

ELISA with EDII dimer interface antibodies. Two antibodies that bind to the interfaces of adjacent E protein dimers, kindly provided by M. Diamond, Washington University (40), were used to determine whether E85 protein expressed from the VRP vector forms E dimers. ELISA plates were coated with mouse monoclonal antibodies DV2-46 and DV2-58 overnight at 4°C. The plates were washed with Tris-buffered saline with 0.2% Tween 20 (TBST wash buffer) and blocked with 5% skim milk in Tris-buffered saline with 0.05% Tween 20 (TBST blocking buffer) for 1 h at 37°C. Serially diluted supernatant and cell lysate from DENV2 E85-

VRP-infected Vero cells in TBST blocking buffer were then added to each well and incubated for 1 h at 37°C. After washing 3 times with TBST wash buffer, the plates were incubated for 1 h at 37°C with 100 ng of EDIII-reactive human MAb 1M23 (50). The plates were washed and incubated with alkaline phosphatase-conjugated goat anti-human antibody (Sigma) and then washed 3 times with TBST wash buffer and developed by adding *p*-nitrophenyl phosphate substrate (Sigma). The optical density (OD) was measured at 405 nm using a spectrophotometer. Serially diluted recombinant E80 protein purchased from Hawaii Biotech was used as a positive control.

DENV neutralization assays. DENV serotype-specific neutralizing antibodies in immunized macaque sera were quantified using a flow cytometry-based neutralization assay on Vero cells, as described previously (28, 66, 67). The assay has been previously validated by head-to-head comparison with a classical plaque reduction neutralization assay (PRNT) in a study using a panel of WHO reference sera from all 4 serotypes and convalescent-phase sera from patients (66). It has the advantage of reduced performance time from 1 week to 3 days, a higher-throughput format, and the ability to measure the neutralization of clinical isolates and viral strains that have not been adapted to grow on Vero or BHK cells and that do not plaque well in the PRNT. In the flow-based assay, the ability of immune serum to neutralize the infectivity of DENV on Vero cells is measured at 24 h postinfection (p.i.) by enumerating cells that are positive for intracellular staining of DENV prM or E protein by flow cytometry. The strains of DENV used in this assay were reference strains provided by WHO representing each serotype, as specified above. Where indicated, strains representing genotypes I through IV within serotype 3 were used. The controls included in each assay were (i) mock-infected cells, (ii) cells infected with DENV in the absence of immune sera or with preimmune sera, and (iii) cells infected with DENV preincubated with a reference DENV immune serum. The percent neutralization for each dilution was defined as the reduction in the number of E-antigen-positive cells in the test sera compared with the number of positive cells in the virus-only control. The neutralization titer (Neut₅₀ titer) for each serum sample was expressed as the reciprocal of the titer that reduced infection to ≤50% (50% effective concentration [EC₅₀]), calculated using nonlinear regression analysis, sigmoidal model.

VEE neutralization assay. VEE-specific neutralization titers in sera from VRP-immunized animals were determined using a flow cytometry-based neutralization assay. Briefly, BHK cell monolayers were seeded in 24-well plates at a density of 10⁵ cells per well. Control or immune sera were diluted 2-fold with alpha-MEM containing 1% bovine serum albumin, and each dilution was mixed with an equivalent volume of a VRP expressing green fluorescent protein (GFP-VRP) to give a multiplicity of infection (MOI) of 0.1 in a total volume of 110 µl. The mixture was incubated for 1 h at 37°C in 5% CO₂ before transferring it to BHK cell monolayers in 24-well plates. Each serum dilution was tested in duplicate wells. After 1 h of adsorption, complete medium was added, and the plates were incubated for 24 h at 37°C. To enumerate infected cells, the monolayers were washed with PBS, trypsin treated, washed again with PBS, and fixed in 2% paraformaldehyde. The cells were analyzed by flow cytometry in a Cyan (Becton, Dickinson). The percent neutralization for each dilution was defined as the reduction in the number of GFP-expressing cells in the test sera compared with the number of positive cells in the VRP control. The neutralization titer for each serum sample was expressed as the reciprocal of the highest dilution of serum that neutralized the GFP-VRP by ≥50% (NEUT₅₀).

Macaque immunizations and virus challenge. Young adult rhesus macaques (2 to 8 years of age) seronegative for dengue virus and VEE were housed at the Caribbean Primate Research Center facilities, University of Puerto Rico, San Juan, Puerto Rico. All procedures were reviewed and approved by the Institute's Animal Care and Use Committee at Medical Sciences Campus, University of Puerto Rico (IACUC-UPR-MSC), and performed in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) (Animal Welfare

Assurance number A3421; protocol numbers, 7890108, 7890208, 7890209, and 7890210). In addition, steps were taken to ameliorate suffering in accordance with the recommendations of the Weatherall report, *The Use of Nonhuman Primates in Research*. For instance, to ameliorate animal suffering, all procedures were conducted under anesthesia using ketamine at 10 to 20 mg/kg of body weight intramuscularly (i.m.), as approved by the IACUC. Anesthesia was delivered in the caudal thigh using a 23-gauge sterile syringe needle. Continued monitoring was provided by trained veterinarians at the Animal Research Center. During the time of the protocol, the animals were under the environmental-enrichment program of the facility, also approved by the IACUC. Vaccines were formulated in PBS with 1% human serum albumin. The 4 components of the tetravalent formulation were stored separately at −80°C and mixed immediately before administration to the animals. Groups of 6 macaques were immunized by intradermal injection into the upper arm with 0.5 ml/arm of VRP vaccine at a dose of 10⁸ IU for the monovalent DENV3 vaccine and 10⁸ IU of each DENV1, DENV2, and DENV3 component and 2 × 10⁸ IU of the DENV4 component of the tetravalent formulation. Macaques received 3 vaccine doses (weeks 0, 7, and 25) in the monovalent vaccine study or 2 vaccine doses (weeks 0 and 6) in the tetravalent study. The animals were bled periodically on the day before each immunization and at 2- or 3-week intervals postimmunization. To determine protective efficacy, in study week 41 (monovalent study) or week 24 (tetravalent study), all immunized macaques, along with groups of 4 unvaccinated control animals, were challenged with 10⁵ PFU of DENV3 Sleman/78 (monovalent study) or 10⁵ PFU of either DENV1 WP, DENV2 NGC, DENV3 Sleman/78, or DENV4-814669 (tetravalent study). The animals were bled every day for 10 days postchallenge and on day 28 after challenge.

Expression and purification of DENV EDIII. Recombinant EDIII protein fused at the N terminus to maltose binding protein (MBP-EDIII) was produced as described previously (65). Domains III of the E protein from DENV2 strain NGC (residues 297 to 399) and DENV3 strain UNC 3006 (residues 295 to 398) were expressed as MBP fusion proteins in *Escherichia coli* DH5α (Invitrogen) and purified using amylose resin affinity chromatography (NEB) as described previously (65).

Depletions of EDIII-reactive antibodies in macaque serum. MBP-EDIII and MBP control depletions were done as described previously (65). Briefly, purified MBP-EDIII and MBP control were equilibrated in amylose column buffer (NEB). Amylose resin was incubated with 300 µg of recombinant protein in the column buffer. After overnight incubation at 4°C, the resin was washed 3 times with column buffer and 3 more times with PBS to remove unbound recombinant protein. After blocking with 5% normal mouse serum (NMS) or 1% bovine serum albumin (BSA) in PBS, the resin was incubated with 1.5 ml of macaque serum diluted 1:5 in PBS for 2 h at 37°C. The amylose resin was removed by pelleting, and the serum, depleted of EDIII-reactive antibodies, was subjected to another 4 or 5 depletion cycles until complete depletion was confirmed by the absence of EDIII-reactive antibodies by ELISA, using plates coated with MBP and MBP-EDIII. EDIII and control depleted sera were then tested for the ability to neutralize DENV *in vitro*, as described above.

Detection of viremia by immunofocus assay. The presence of virus in serum samples collected daily for 10 days postchallenge was determined by incubation on Vero cell monolayers, followed by immune detection of virus foci using an enzyme-labeled dengue virus MAb, as described previously (68). Briefly, Vero-81 cells were seeded in 24-well plates, and subconfluent monolayers were incubated the next day in triplicate wells with 200 µl of each postchallenge serum sample at dilutions of 1:2 and 1:4 or 1:2.5 and 1:5. After a 1-h incubation on a rocker at 37°C, the monolayers were overlaid with 1 ml 1.0% methylcellulose in Opti-MEM (Gibco) supplemented with 2% fetal bovine serum (FBS) (Cellgro) and antibiotic mixture (Gibco; Antibiotic-Antimycotic). After 4 to 5 days at 37°C, 5% CO₂, the overlay was removed, and the cells were washed with PBS, fixed in 80% methanol-1× PBS, and air dried. To visualize the virus foci, the fixed monolayers were blocked for 10 min with blocking buffer (5% in-

FIG 1 *In vitro* expression of DENV E protein in VRP-infected BHK cells. BHK-21 cells grown in 24-well plates were mock infected or infected at an MOI of 10 with VRP expressing DENV3 prME or E85. Culture media and cell lysates in each well were harvested at 19 h postinfection and analyzed for the presence of the E protein by nonreducing SDS-PAGE, followed by Western blotting. Approximately 33% of the cell lysate and 7% of the culture medium per well was loaded in each lane. Electrophoresed proteins were transferred onto polyvinylidene fluoride membranes and probed with mouse Mab 4G2. Following incubation with goat anti-mouse IgG-HRP, membranes were developed using ECL substrate. M, mock infected; prME, DENV3 prME-VRP; E85, DENV3 E85-VRP. Bands migrating at the expected sizes corresponding to full-length E protein (E) and C-terminally truncated 85% of E (E85) are indicated.

stant milk in PBS), followed by incubation with anti-flavivirus Mab 4G2 diluted 1:60 in blocking buffer for 1 h at 37°C. The wells were washed with PBS and incubated with HRP-conjugated goat anti-mouse Ab (Sigma) diluted 1:500 in blocking buffer for 1 h at 37°C. The plates were washed once in PBS, and foci were developed by the addition of 150 μ l/well of TrueBlue HRP substrate (KPL). Foci were counted on a light box, and viral titers were calculated by standard methods.

Statistical analysis. Antibody titers were evaluated for statistically significant differences by the Mann-Whitney test (INSTAT; GraphPad, San Diego, CA). A *P* value of <0.05 was considered significant.

RESULTS

***In vitro* characterization of dengue virus proteins expressed by VRP.** BHK-21 cell cultures infected with prME-VRP or E85-VRP or mock infected were harvested at 19 h postinfection and examined for the expression of dengue virus E protein by SDS-PAGE, followed by Western blotting. PVDF membranes were probed

with Mab 4G2. **Figure 1** shows the results from serotype 3 constructs, which are representative of the other three serotypes. The C-terminally truncated E85 expressed from E85-VRP and the full-length E expressed from prME-VRP ran at the expected apparent molecular sizes. E85 showed higher expression levels than full-length E in both cell lysates and culture media. In the culture medium lanes, the bands were lighter than those in the cell lysate lanes, reflecting the smaller fraction (~7%) of the total infection media examined (versus 33% of cell lysates).

To determine whether VRP-expressed E85 formed dimers, serial dilutions of cell lysates and culture supernatants of BHK cells infected with DENV2 E85-VRP were tested by ELISA for the ability to bind to mouse MAb DV2-58 and DV2-46, which bind to the DENV2 dimer interface (40). **Figure 2** shows that VRP-expressed E85, whether cell associated or secreted, has high reactivity with both MAb, indicating their ability to form dimers.

Comparison of the immune responses induced in macaques by vaccination with VRP expressing different dengue virus antigens and infection with dengue virus. The neutralizing-antibody responses to immunization were examined in terms of their magnitude, breadth, antigenic sites targeted, and ability to protect from DENV3 challenge. In addition, we compared the vaccine-induced antibody responses to those induced by the DENV3 challenge of naïve animals as a surrogate for natural infection.

Monovalent VRP vaccines induce neutralizing-antibody responses and protection from challenge. In this monovalent vaccine study, two groups of 6 macaques each were immunized with 3 doses of 10^8 IU of DENV3 prME-VRP or DENV3 E85-VRP at study weeks 0, 7, and 25. At 14 weeks after the last immunization, the 12 immunized monkeys and 4 unimmunized controls were challenged with DENV3. Sera collected periodically after each of 3 immunizations with DENV3 prME-VRP or E85-VRP were analyzed for neutralizing antibodies to DENV3. **Table 1** shows the Neut_{50} titers for each individual macaque after the first, second, and third immunizations and the geometric mean titer (GMT) for each vaccine group. The E85 configuration resulted in significantly higher neutralizing-antibody titers to the serotype 3 virus than prME. Seven weeks after dose 1, macaques immunized with prME-VRP did not show detectable neutralizing-antibody titers, while 5 of 6 animals immunized with E85-VRP had Neut_{50} titers

FIG 2 E85 binds to MAb that recognize only the interface of adjacent E dimers. Mouse MAb DV2-58 and DV2-46, which bind to the DENV2 dimer interface, were used to coat ELISA plates. Serially diluted supernatants and cell lysates from DENV2 E85-VRP or mock-infected Vero cells harvested 24 h p.i. were then added to the wells in duplicate. E85 binding was detected with EDIII-reactive human Mab 1M23, followed by incubation with alkaline phosphatase-conjugated goat anti-human antibody and *p*-nitrophenyl phosphate substrate. The data points represent mean $\text{OD}_{405} \pm$ standard deviations (SD). dln, dilution.

TABLE 1 Neut₅₀ against DENV serotype 3^a after the 1st, 2nd, and 3rd immunizations^b with monovalent DENV3-VRP vaccines

Vaccine	Monkey ID ^c	Neut ₅₀ ^f			
		7 wk after dose 1	3 wk after dose 2	18 wk after dose 2	14 wk after dose 3 prechallenge
DENV3	AJ97	<20	80	23	32
prME-VRP ^c	AH06	<20	38	22	<20
	M654	<20	46	<20	24
	M745	<20	46	<20	20
	72V	<20	58	22	21
	98O	<20	35	<20	<20
GMT ^d		5	49	11	14
DENV3	AI15	51	244	84	280
E85-VRP ^c	AH52	<20	70	29	58
	AM41	34	425	134	203
	M635	20	176	50	61
	M636	30	161	42	146
	M630	28	467	30	48
GMT		23	214	52	106

^a The DENV3 used in the neutralization assay was reference strain CH53489.^b Dose 1, week 0; dose 2, 7 weeks after dose 1; dose 3, 18 weeks after dose 2.^c Macaques were immunized by intradermal injection in the upper arm with 10⁸ IU of VRP vaccine.^d For calculations of GMTs, titers of <20 were assigned a value of 5.^e ID, identifier.^f Neut₅₀ titers are significantly different between prME-VRP and E85-VRP groups after doses 1, 2, and 3 ($P < 0.05$ at every time point by Mann-Whitney test).

equal to or above 20. Three weeks after the second dose, all the animals in both groups had a significant increase in titer, with GMTs of 49 for prME and 214 for E85. When measured 18 weeks after the second dose, Neut₅₀ titers had dropped; however, all

E85-VRP-immunized monkeys had titers above 20, with a GMT of 52, while 3 out of 6 prME-VRP-immunized monkeys had titers above 20. After a third dose, the titers increased in both groups, and at the time of challenge, 14 weeks after the last immunization, 4 of 6 macaques immunized with the prME construct had modest titers ranging from undetectable (<20) to 32, while all macaques immunized with E85 had titers ranging from 48 to 280, with a GMT of 106 (Table 1). The improved immunogenicity of E85 over prME was also demonstrated for serotype 2 in a smaller experiment with a similar immunization schedule but only two macaques per group. After the first dose, only E85-VRP-immunized animals had detectable neutralizing-antibody titers (a Neut₅₀ of 40 for each animal). After the second dose, E85-VRP induced titers of 160 and 320, while prME-VRP titers were 20 and 80. After the third dose, the E85-VRP titers were 320 and 320, while the prME titers were 40 and 40.

To determine the abilities of the vaccine candidates to protect from infection, vaccinated macaques, along with 4 unvaccinated controls, were challenged with 10⁵ PFU of DENV3 strain Sleman/78 live virus at 14 weeks after the last immunization. Because macaques do not develop signs of disease after DENV infection but do develop a low-level viremia during the 10 days immediately following the challenge, protective efficacy was determined by the ability of the vaccine to reduce the duration and titer of peak viremia after challenge. Serum viremia was determined by immunofocus assay in samples collected daily for 10 days. Table 2 shows the titers of DENV, expressed as log₁₀ focus-forming units (FFU)/ml, in the serum of each macaque. All the unvaccinated control macaques had viremia between days 2 and 8 postchallenge, with titers ranging from 2.57 to 3.89 log₁₀ FFU/ml. Monkeys immunized with prME-VRP were partially protected. Four out of 6 had no detectable virus, and the other 2 showed viremia of shorter duration. In contrast, all the animals immunized with E85-VRP were protected, as indicated by undetectable viremia (limit of de-

TABLE 2 Viremia and Neut₅₀ titers before and after DENV3 challenge of macaques immunized with monovalent DENV3-VRP^a

Vaccine group	Monkey ID	Viremia ^b (log ₁₀ FFU/ml) on day postchallenge:										Neut ₅₀ anti-DENV3	
		1	2	3	4	5	6	7	8	9	10	Before challenge ^c	28 days after challenge
Unvaccinated controls	ID3	—	3.50	3.60	3.40	3.00	—	—	—	—	—	<20	235
	IE9	—	—	2.64	2.57	2.87	3.38	3.59	2.79	—	—	<20	357
	6D5	—	3.77	3.89	3.09	2.87	—	—	—	—	—	<20	180
	50T	—	—	—	—	—	3.00	2.69	—	—	—	<20	55
DENV3 prME-VRP	AJ97	—	—	—	—	—	—	—	—	—	—	32	20,480
	AH06	—	—	—	—	—	—	—	—	—	—	<20	4,321
	M654	—	—	—	—	—	—	—	—	—	—	24	2,477
	M745	—	—	—	—	—	—	—	—	—	—	20	4,125
	72V	—	—	3.17	2.69	—	—	—	—	—	—	21	17,362
	98O	—	—	2.57	2.94	—	—	—	—	—	—	<20	3,272
DENV3 E85-VRP	AI15	—	—	—	—	—	—	—	—	—	—	280	12,550
	AH52	—	—	—	—	—	—	—	—	—	—	58	20,480
	AM41	—	—	—	—	—	—	—	—	—	—	203	9,954
	M635	—	—	—	—	—	—	—	—	—	—	61	20,480
	M636	—	—	—	—	—	—	—	—	—	—	146	11,981
	M630	—	—	—	—	—	—	—	—	—	—	48	20,480

^a Monkeys were challenged 14 weeks after dose 3.^b Viremia was quantified by immunofocus assay, which measured infectious virus on Vero cells. —, below the level of detection of 10 FFU/ml.^c Prechallenge Neut₅₀ titers were determined on the day of challenge.

FIG 3 Serotype-specific and cross-reactive neutralizing-antibody titers (Neut_{50}) in macaques after monovalent DENV3-VRP immunization and after DENV3 infection. Three weeks after dose 2, sera from macaques immunized with DENV3 prME-VRP (A) or DENV3 E85-VRP (B), and 4 weeks postchallenge, sera from unimmunized control macaques (C) were tested for neutralization against DENV reference strains from serotypes 1, 2, 3, and 4: DENV1, West Pac 74; DENV2, S-16803; DENV3, CH53489; DENV4, TVP-360 (DV1 to DV4, respectively). Each symbol represents the Neut_{50} titer of an individual macaque. The GMT is represented by a horizontal bar. Dashed lines, lower limit of detection. Samples with Neut_{50} values below 10 were assigned a value of 5.

tection, 10 FFU/ml). The last 2 columns in Table 2 show Neut_{50} titers before challenge and 28 days after challenge. From these results, it was not possible to determine a minimum prechallenge titer that would correlate with protection. While the prechallenge titers of the 2 animals in the prME-VRP group that showed detectable postchallenge viremia (macaques 72V and 980) were at or below the level of detection, other macaques with low titers ranging from <20 to 32 showed no postchallenge viremia. All the vaccinated animals had a very strong recall response after the challenge, with Neut_{50} titers that were 36 to 121 times higher than those in the unvaccinated controls at this time point. This suggests that the vaccine did not induce sterilizing immunity and also indicates the strong priming effect of the VRP vaccine. The convalescent-phase serum neutralization titers in the unvaccinated control animals at 28 days postinfection ranged from 55 to 357. These titers are comparable to those induced after 2 doses of E85-VRP immunization, which ranged from 70 to 467 at 21 days after the second dose.

In summary, both DENV3-VRP vaccines induced neutralizing antibodies and protection from DENV3 challenge-induced viremia. However, E85-VRP induced a faster and stronger neutralizing-antibody response, which was consistent with better protection from challenge than prME-VRP. In addition, our data indicate that the priming afforded by the VRP vaccines is sufficient, not only to significantly reduce the replication of challenge virus, but also to prime a strong anamnestic response consistent with long-term immunity.

Breadth of the neutralizing-antibody response. To further characterize the quality of the antibody response induced by DENV3 prME-VRP, E85-VRP, and live DENV3 infection, we measured the induction of serotype cross-reactive neutralizing antibodies. The virus strains used in the neutralization assay were WHO reference strains representing each serotype, as described in Materials and Methods. Neutralization titers to all 4 serotypes were determined in sera collected 3 weeks after the second VRP immunization. DENV3 prME-VRP preferentially induced titers to serotype 3 virus, but also induced detectable neutralizing-antibody titers to the heterologous serotypes in all the animals, with Neut_{50} GMTs of 23, 39, 58, and 24 to serotypes 1, 2, 3, and 4, respectively (Fig. 3A). In contrast, E85-VRP immunization induced strong serotype 3-specific antibodies, with undetectable neutralizing antibodies to the other three serotypes (Fig. 3B). The breadth of the antibody response after live DENV infection was

also examined as a control representing live-virus immunization/infection. Macaque sera collected 4 weeks after infection with DENV3 in unimmunized control animals were able to neutralize the homologous serotype 3 virus with high Neut_{50} titers but also had cross-reactive antibodies with neutralizing activity to serotype 1, 2, and 4 viruses (Fig. 3C).

Due to the serotype-specific nature of the neutralizing-antibody response to the E85-VRP vaccine, and based on recent observations indicating that genotypic variation within each serotype may be important in protective immunity (37, 42, 69, 70), we examined the influence of intraserotype genotypic differences on vaccine performance. For this, we tested the ability of DENV3 E85-VRP macaque sera to neutralize DENV3 strains representing genotypes I to IV (Fig. 4). We found that the vaccine-induced antibodies were able to neutralize with equal potency viruses from the vaccine strain genotype (GIII) and from the other genotypes (GI, GII, and GIV).

In summary, macaque antibody responses to prME-VRP and live virus were characterized by dominant serotype-specific neutralizing antibodies and lower but detectable cross-reactive antibodies to the other serotypes. In contrast, antibodies to E85-VRP neutralized only serotype 3 virus isolates, including strains representing all DENV3 genotypes (I to IV).

FIG 4 Breadth of serotype-specific neutralizing-antibody response after monovalent DENV3 (GIII) E85-VRP immunization. Six weeks after dose 2, sera from DENV3 E85-VRP-immunized animals were tested for neutralization against strains from different genotypes within serotype 3: DENV 3043 for GI, CH53489 for GII, UNC 3002 for GIII (homologous to the vaccine strain), and DENV3PR77 for GIV. Each symbol represents the Neut_{50} titer of an individual macaque. The GMT is represented by a horizontal bar. Dashed lines, lower limit of detection.

FIG 5 Contribution of DENV3 EDIII-binding antibodies to neutralization. Macaque immune sera were depleted of DENV3 EDIII-binding antibodies by incubation with EDIII-MBP or control MBP protein bound to amylose beads. Neutralizing-antibody titers to DENV3 were determined in control-depleted and EDIII-depleted sera for each animal. The horizontal bars represent geometric mean titers for each depletion group. (A) Sera from macaques 3 weeks after the second immunization with DENV3 prME-VRP. (B) Sera from macaques 3 weeks after the second immunization with DENV3 E85-VRP. (C) Sera from macaques 4 weeks after DENV3 infection. Complete depletion of EDIII-reactive antibodies was confirmed by EDIII ELISA. The data are representative of one of three experiments. **, $P < 0.05$ by the Mann-Whitney test.

Contribution of EDIII-binding antibodies to the neutralizing response. Previous studies have demonstrated that mice challenged with DENV develop high levels of neutralizing antibodies that bind to EDIII, whereas humans exposed to natural dengue or West Nile virus infections develop neutralizing-antibody responses that mainly target epitopes on EDI and EDII (47, 65, 71, 72). The major antigenic sites targeted by neutralizing antibodies induced by the VRP vaccines in macaques were compared to those induced by virus infection with respect to EDIII specificity. DENV3 EDIII-binding antibodies were depleted from the sera of DENV-immune macaques using recombinant EDIII-MBP or control MBP protein bound to amylose resin beads. Depletion was monitored by EDIII-MBP binding ELISA, and complete depletion (the ELISA OD at 405 nm [OD_{405}] was <0.1 at 1:10 serum dilution) was confirmed after 6 depletion cycles.

Figure 5 shows Neut₅₀ titers of control MBP-depleted and DENV3 EDIII-MBP-depleted sera from DENV-immune macaques. In sera collected after two immunization doses with DENV3 prME-VRP, neutralizing-antibody titers did not change after EDIII-binding antibodies were depleted (Fig. 5A). In contrast, sera from DENV3 E85-VRP macaques at the same time point showed a significant reduction in neutralization titers after EDIII-binding antibodies were depleted, suggesting that a large proportion of neutralizing antibodies were directed to EDIII (Fig. 5B). Control monkeys infected with DENV3 had neutralization titers that did not change after depletion of EDIII-binding antibodies (Fig. 5C). This was consistent with reports that EDIII antibodies contribute little to the neutralizing response after a natural infection in humans (48, 65) and suggested that DENV infection in macaques stimulates a dengue virus-specific antibody repertoire similar to that in humans. These depletion studies also suggest that E antigen can induce a qualitatively different antibody response, depending on its configuration, and that the quality of the immune response elicited by prME-VRP more closely resembles the immune response after a natural DENV infection than that elicited by E85-VRP.

Tetravalent VRP using E85 antigen configuration in macaques. We chose in this study to construct and evaluate a

tetravalent vaccine expressing the E85 configuration. Our rationale was to construct a tetravalent formulation in which each serotype component would induce strong serotype-specific neutralizing antibodies and in which EDIII epitopes for each serotype would make a significant contribution. In addition, the absence of prM would reduce the fraction of weakly neutralizing, cross-reactive, potentially enhancing antibodies that map to prM.

Neutralizing-antibody responses. To assess whether a tetravalent cocktail of VRP expressing E85 from each serotype would induce a balanced immune response against all 4 serotypes, 16 young adult rhesus macaques were immunized at 0 and 6 weeks with a cocktail containing 1×10^8 IU DENV1 E85-VRP, 1×10^8 IU DENV2 E85-VRP, 1×10^8 IU DENV3 E85-VRP, and 2×10^8 IU DENV4 E85-VRP. The vaccine components were mixed at a ratio of 1:1:1:2 based on results in mice, where we found that DENV4 E85-VRP, as a monovalent vaccine and in a tetravalent cocktail with equal doses of each serotype, was less immunogenic than the other 3 serotypes. Adjusting the dose of DENV4 E85-VRP in the tetravalent cocktail resulted in higher DENV4 neutralization titers and more balanced responses (White, unpublished). The vaccine regime in this tetravalent VRP study was reduced to two instead of three immunizations, since in the monovalent study, two doses were sufficient to induce 100% seroconversion.

Table 3 shows the 50% neutralization titers of individual macaques and GMTs after the first and second doses and before challenge. Preimmunization titers were <10 in all animals tested (data not shown). After one dose, 13 of 16 macaques had detectable neutralizing antibodies to at least 3 serotypes, with Neut₅₀ GMTs of 20, 41, 10, and 34 to serotypes 1, 2, 3, and 4, respectively. After the second immunization, all 16 macaques had neutralizing-antibody titers against all 4 serotypes, with GMT titers of 283, 446, 202, and 284 against serotypes 1, 2, 3, and 4, respectively (Table 3). Although there was animal-to-animal variation in the titers, all animals had robust neutralizing-antibody titers against all 4 serotypes after 2 doses, with titers to DENV2 being higher than those against other serotypes in 9/16 animals. Although the titers had dropped by the time of challenge, all the macaques had detectable

TABLE 3 Neutralizing-antibody titers against DENV serotypes 1 to 4 in macaques after immunization with 2 doses^a of tetravalent E85-VRP^b

Macaque ID	Neut ₅₀ against:											
	6 wk after dose 1				2 wk after dose 2				18 wk after dose 2 (prechallenge)			
	DENV1	DENV2	DENV3	DENV4	DENV1	DENV2	DENV3	DENV4	DENV1	DENV2	DENV3	DENV4
2H9	<10 ^c	22	<10	23	274	735	212	292	112	67	36	101
9H4	50	84	35	48	359	1516	297	264	29	73	93	48
8E7	<10	<10	<10	<10	85	63	154	102	53	24	81	23
6F5	32	38	<10	36	201	337	91	89	44	106	58	35
6E1	25	18	25	27	187	167	128	73	22	50	11	56
6B1	11	<10	15	23	142	55	97	63	23	17	14	24
1C4	116	101	38	153	395	938	218	726	207	97	13	140
9B4	<10	15	<10	<10	370	306	168	337	44	174	168	192
3C1	13	13	11	31	300	378	219	462	35	40	24	48
4F3	77	84	35	88	469	993	341	223	48	331	30	323
4C7	12	<10	16	94	308	232	134	660	61	56	14	203
0G6	64	29	<10	97	215	454	170	684	73	101	19	176
3G8	176	111	77	58	674	1096	530	278	232	193	657	215
BA35	30	77	77	38	296	619	276	522	453	170	38	176
AZ99	61	97	35	70	458	1706	313	445	186	329	58	143
BA10	88	35	44	97	307	865	241	778	80	88	44	134
GMT	28	29	18	40	283	446	202	284	71	88	41	97
% Seroconversion ^d	81	81	69	88	100	100	100	100	100	100	100	100

^a Dose 1, week 0; dose 2, 6 weeks after dose 1; challenge, 18 weeks after dose 2.

^b TV-E85-VRP was formulated by mixing 1×10^8 IU each of DENV1-, -2-, and -3-E85-VRP and 2×10^8 IU of DENV4-E85-VRP.

^c Samples with Neut₅₀ titers lower than 10, the lowest serum dilution tested, were assigned a titer of 5 for calculation of the GMT.

^d Seroconversion was defined as a Neut₅₀ titer of ≥ 10 .

neutralizing antibodies against all 4 serotypes, with GMTs of 71, 88, 41, and 97 to serotypes 1, 2, 3, and 4, respectively.

The bottom row in Table 3 shows the percentage of animals with neutralizing antibodies to each serotype after the first and second immunizations and on the day of challenge. After one dose, 81% of the animals had neutralizing antibodies to 3 or more serotypes, and 56% had neutralizing antibodies to all 4 serotypes. After the second dose, all the animals had neutralizing antibodies to all 4 serotypes. Four months later, although titers had waned in some animals, 16 out of 16 retained neutralizing antibodies against all 4 serotypes.

As monovalent DENV3 E85-VRP vaccination induced predominantly serotype-specific, EDIII-targeted neutralizing antibodies, studies were done to test if the tetravalent vaccine stimulated 4 separate populations of neutralizing antibodies that bound

to EDIII of each serotype. Immune sera from monkeys that received the E85 VRP tetravalent vaccine were depleted of DENV3 EDIII-binding antibodies and tested for the ability to neutralize each serotype (Fig. 6). Depletion of DENV3 EDIII-reactive antibodies resulted in a large decrease in DENV3 neutralization, a slight decrease in DENV1 neutralization, and no change in the ability to neutralize serotypes 2 and 4. These results suggest that as in the monovalent DENV3 E85-VRP vaccine, the DENV3 E85-VRP component in the tetravalent vaccine stimulates a distinct population of EDIII-reactive antibodies with serotype-specific neutralization. It is likely that the tetravalent vaccine will elicit four monovalent EDIII responses. However, since we depleted only with DENV3 EDIII, we cannot conclude unequivocally that the other 3 components of the immune response are also specific. The reduced ability of the DENV3 EDIII-depleted immune sera to

FIG 6 Contribution of DENV3 EDIII-binding antibodies to neutralization in macaques immunized with tetravalent DENV E85-VRP. Macaque immune sera were depleted of DENV3 EDIII-binding antibodies by incubation with EDIII-MBP or control MBP protein bound to amylose beads. Neutralizing-antibody titers to each serotype were determined. (A) Anti-DENV3. (B) Anti-DENV1. (C) Anti-DENV2. (D) Anti-DENV4. The horizontal bars represent geometric mean titers for each depletion group. Complete depletion of EDIII-reactive antibodies was confirmed by EDIII ELISA. *, $P = 0.0286$, and **, $P = 0.0031$ by the Mann-Whitney test. Within each graph, each symbol type refers to the same animal.

TABLE 4 Postchallenge viremia and Neut₅₀ titers before and after macaque challenge with DENV1, -2, -3, and -4

Monkey ID	Challenge serotype	Vaccine group	Viremia ^a (log ₁₀ FFU/ml) on day postchallenge:										Neut ₅₀ titer ^b	
			1	2	3	4	5	6	7	8	9	10	Prechallenge	Day 10 postchallenge
6B1	DENV1	Immunized	—	—	1.52	—	—	—	—	—	—	—	23	19,953
1C4			—	—	—	—	—	—	—	—	—	—	207	13,146
2H9			—	1.23	—	—	—	—	—	—	—	—	112	13,146
3G8			—	—	—	—	—	—	—	—	—	—	232	14,423
4E6		Controls	2.09	2.96	2.22	0.90	—	—	—	—	—	—	<10	446
IF5			1.23	1.40	—	1.52	2.15	1.23	—	—	—	—	<10	367
5E5			2.35	2.89	2.45	1.23	1.23	—	—	—	—	—	<10	711
2F6			—	2.65	2.45	1.23	—	0.90	—	—	—	—	<10	134
6F5	DENV2	Immunized	—	—	—	—	—	—	—	—	—	—	106	>20,480
6E1			—	2.18	1.83	—	—	—	—	—	—	—	50	>20,480
9B4			—	1.23	—	—	—	—	—	—	—	—	174	>20,480
BA35			—	—	1.23	—	0.90	—	—	—	—	—	170	>20,480
3F3		Controls	—	2.09	1.40	1.40	0.90	1.23	0.90	—	—	—	<10	608
9E2			—	1.52	1.23	1.40	1.52	2.12	2.28	—	—	—	<10	413
BC19			—	2.22	2.07	1.23	—	—	—	—	—	—	<10	1,126
OHI			—	2.58	1.40	—	—	—	—	—	—	—	<10	1,590
8E7	DENV3	Immunized	—	—	—	—	—	—	—	—	—	—	81	5,201
4F3			—	—	—	—	—	—	—	—	—	—	30	10,921
3C1			—	—	—	—	—	—	—	—	—	—	24	19,046
9H4			—	—	—	—	—	—	—	—	—	—	93	4,996
2G5		Controls	—	1.23	3.01	2.67	2.12	2.54	—	—	—	—	<10	104
3H8			—	—	0.90	1.52	1.40	1.70	—	—	—	—	<10	263
4H9			—	—	2.41	1.62	1.23	1.40	—	—	—	—	<10	225
8F7			—	—	1.52	1.23	—	—	—	—	—	—	<10	1017
4C7	DENV4	Immunized	—	—	—	—	—	—	—	—	—	—	203	3,272
0G6			—	—	—	—	—	—	—	—	—	—	176	10,426
AZ99			—	—	—	—	—	—	—	—	—	—	143	2,433
BA10			—	—	—	—	—	—	—	—	—	—	134	13,146
9G9		Controls	2.09	1.52	1.23	—	—	—	—	—	—	—	<10	233
BD57			2.22	2.09	1.83	—	—	—	—	—	—	—	<10	353
6F8			2.35	2.07	1.23	—	—	—	—	—	—	—	<10	185
6G0			0.90	1.83	1.23	1.30	1.70	1.40	1.23	—	—	—	<10	46

^a Viremia was quantified by immunofocus assay, which measured infectious virus on Vero cells. —, below the level of detection of 8 FFU/ml.

^b Neut₅₀ titers against the serotype of the challenge virus.

neutralize DENV1 suggests that the vaccine targets subcomplex cross-reactive epitopes on EDIII and is consistent with the observation that strains from serotypes 1 and 3 are closer phylogenetically to each other than to serotypes 2 and 4 (92).

Protection from postchallenge viremia after 2 doses. To determine whether the tetravalent VRP vaccine would protect against infection with each DENV serotype, the 16 immunized macaques were randomly assigned to 4 challenge groups (4 animals/group). Another 16 unimmunized macaques were divided into 4 challenge groups (4 animals/group) and used as controls. This resulted in four challenge groups of 8 macaques each (4 immunized and 4 unimmunized). The macaques in groups 1, 2, 3, and 4 were challenged in week 18 postboost with 10⁵ FFU of DENV1, DENV2, DENV3, and DENV4, respectively. The animals were bled daily for 10 days and then again on day 28. Table 4 shows DENV titers in the serum of each animal during the 10 days following challenge. The values represent log₁₀ FFU/ml as determined by immunofocus assay. Of the 8 macaques challenged with DENV1, the 4 controls had viremia lasting from 3 to 5 days, with peak titers of 2.96, 2.15, 2.89, and 2.65 log₁₀ FFU/ml. Two of the 4 immunized macaques had no detectable viremia, while the other 2 animals showed low-level viremia (1.52 and 1.23 log₁₀ FFU/ml)

that lasted 1 day. Of the macaques challenged with DENV2, all the controls had 2 to 6 days of viremia, with peak titers ranging from 2.09 to 2.58 log₁₀ FFU/ml. Three of the immunized monkeys had low-level viremia that lasted 1 or 2 days. In the groups challenged with DENV3, all the control macaques had viremia (peak titers between 1.52 and 3.01) lasting 2 to 5 days, while no viremia was detected in any of the vaccinated animals at any time point. The same was the case for animals challenged with DENV4, where all controls had viremia with peak titers ranging between 1.83 and 2.35 and 3 to 7 days duration, while the 4 immunized animals had no detectable viremia (Table 4).

Prechallenge and postchallenge Neut₅₀ titers against the challenge viruses are shown in Table 4 for each animal. There were 5 immunized macaques that had detectable postchallenge viremia. The three with higher prechallenge Neut₅₀ titers against the challenge virus (2H9, 9B4, and BA35) had very low viremia (at the level of detection) that lasted only 1 day. Another macaque with breakthrough viremia (6B1) had a homologous Neut₅₀ titer of 23 and low viremia lasting 1 day. Macaque 6E1 had a prechallenge Neut₅₀ titer of 50 and peak viremia of 2.18.

To measure the ability of the vaccine to induce a strong anamnestic response, Neut₅₀ titers were measured at day 10 postchal-

FIG 7 Effect of anti-vector immunity on the ability of the second VRP dose to boost DENV neutralizing antibodies. Macaques were immunized with 10^8 IU DENV3 prME or E85-VRP at 0 and 7 weeks. (A) At 7 weeks postprime and 6 weeks postboost, animals were bled and neutralizing-antibody titers were determined against VEE. Each symbol represents the Neut₅₀ titer of an individual macaque. The GMT is represented by a horizontal bar. (B) For each monkey, the anti-VEE Neut₅₀ titer at the time of the boost is plotted against the fold increase of the anti-DENV3 Neut₅₀ titer, calculated as the ratio between the titer at 3 weeks after the second dose and 7 weeks after the first dose. (Individual titers are listed in Table 1.)

lenge. Pre- and postchallenge Neut₅₀ titers against the homologous challenge virus are shown in Table 4 for each unvaccinated and TV-E85-VRP-vaccinated animal in the study. There is a strong recall response in all vaccinated animals at only 10 days after challenge with each DENV serotype. In contrast, Neut₅₀ GMTs against the challenge virus in unvaccinated controls were moderate, 353 against DENV1, 656 against DENV2, 281 against DENV3, and 163 against DENV4. We also measured postchallenge neutralization titers to the heterologous serotypes and found a robust recall response, as well. After DENV1 challenge, Neut₅₀ GMTs against serotypes 2, 3, and 4 were 8,174, 1,251, and 993, respectively. After challenge with DENV2, Neut₅₀ GMTs against serotypes 1, 3, and 4 were 4,632, 1,268, and 1,591, respectively. After challenge with DENV3, Neut₅₀ GMTs against serotypes 1, 2, and 4 were 952, 2,187, and 457, respectively. Finally, after challenge with DENV4, Neut₅₀ GMTs against serotypes 1, 2, and 3 were 243, 1,099, and 279, respectively. These results indicate that although the vaccine did not induce sterilizing immunity, it primed a strong anamnestic response, not only to the serotype of the challenge virus, but also to the other 3 serotypes.

Anti-vector immunity. VRP are VEE replicon vectors packaged into particles that have VEE glycoproteins on their surfaces. Although these are single-replication-cycle vectors, anti-VEE antibodies are induced. Our group has shown that mice that have been immunized with VRP expressing one antigen can mount an immune response to a second VRP-expressed antigen that is no different from that of mice that have not been exposed to VRP before. This suggests that in mice, antivector immunity does not interfere with mounting a good immune response to the expressed antigen (N. L. Davis, personal communication). To determine whether this is also the case in macaques, we measured the anti-VEE neutralizing antibodies after the first and second vaccine doses in macaques immunized with prME-VRP or E85-VRP (Fig. 7A). Seven weeks after the first dose, 11 out of 12 macaques had anti-VEE titers ranging from undetectable (<10) to 893, with a GMT of 61. Two macaques had undetectable anti-VEE titers. Six weeks after the second dose, the anti-VEE Neut₅₀ titers ranged from 505 to 2,074, with a GMT of 932, which was significantly higher than the GMT after the first immunization. We then calculated the fold increase in the anti-DENV neutralizing antibodies for each macaque after the boost and plotted the values against the anti-VEE titer at the time of the boost (Fig. 7B). We found that

there was no correlation between anti-vector antibody titers and the fold increase of anti-DENV neutralizing titers between the prime and the boost doses, suggesting that anti-vector antibodies do not significantly interfere with the ability of a second dose to boost.

DISCUSSION

There is an urgent need to accelerate the development of second-generation dengue vaccine candidates. This study demonstrates in an NHP model that the VRP vector is a strong next-generation dengue vaccine platform that provides tetravalent neutralizing-antibody responses after 2 doses and protective efficacy from challenge-induced viremia. The study provides an in-depth evaluation of two VRP-derived dengue vaccine candidates, including a comparison of the qualitative parameters of the antibody responses induced after vaccination versus virus infection.

The first results of a dengue vaccine efficacy study were published recently (19), and they underscore the need not only to accelerate the development of alternative dengue vaccine platforms to LAV, but also to further evaluate current vaccine candidates in preclinical and clinical settings to better characterize quantitatively and qualitatively the vaccine-induced immune responses in a search for better immune correlates of protection. The LAV vaccine candidate used by Sanofi Pasteur, CYD-TDV, was safe in dengue virus-naïve areas and areas of endemicity and induced neutralizing antibodies against all 4 serotypes in most subjects after 3 doses administered in the course of 1 year (18). However, results from a phase 2b proof-of-concept study in Thailand showed unexpectedly low (30%) and insignificant efficacy (19). Surprisingly, there was no measurable efficacy against serotype 2, which was the prevalent serotype during the study. Whether ongoing larger phase 3 trials in different geographic locations will show improved efficacy remains to be determined. Two other tetravalent LAV candidates in earlier phases of clinical testing, NIAID's TetraVax-DV and Inviragen's DENVax, have reported high seroconversion rates in dengue virus-naïve volunteers after only 1 or 2 immunizations (A. Durbin, presented at the American Society of Tropical Medicine and Hygiene 61st annual meeting, Atlanta, GA, 2012; J. E. Osorio, presented at the Third Panamerican Dengue Research Network Meeting, Cartagena, Colombia, 2012). Whether these LAV vaccine candidates will show improved efficacy remains to be demonstrated.

Nonpropagating VEE replicon particles expressing two configurations of DENV3 E antigen, prME and E85, successfully immunized and protected macaques against dengue virus, while anti-vector antibodies did not interfere with a booster immunization. Compared to prME, expression of soluble E85 induced neutralizing antibodies faster and to higher titers, and E85 was more protective in DENV challenge studies. The differences in the magnitude and quality of the neutralizing-antibody responses between DENV3 prME and E85 antigens may have resulted from differences in levels of protein expression and secretion, differences in the quaternary organization of E protein (as soluble dimers or as recombinant subviral particles), and/or whether prM was present. The possible contribution of differences in protein levels was suggested when E protein expression was examined in BHK cells 19 h after VRP infection by Western blotting. We found higher levels of E protein in both cell lysates and culture media of E85-VRP-infected cells than in cells infected with prME-VRP (Fig. 1), suggesting that when equivalent VRP doses are administered, the effective antigen dose may be higher in the E85 configuration.

The quaternary organization of flavivirus E protein has an impact on its antigenic structure (73). In this study, we compared the antibody responses induced by infectious DENV virions, recombinant DENV subviral particles (prME), and soluble E dimers (E85). Coexpression of prM and E of dengue virus and other flaviviruses using heterologous expression systems results in the assembly of recombinant subviral particles (74–79). These subviral particles are smaller than dengue virus virions, have an icosahedral symmetry of $T = 1$ instead of $T = 3$, and may display E proteins with different organizations. However, like virions, they may exist as mature or partially immature particles, depending on the extent of prM cleavage (80, 81). Since we were able to detect E and prM in the supernatant of BHK cells infected with prME-VRP, it is likely that these antigens were present as subviral, partially immature particles. On the other hand, VRP-expressed E85 was secreted as a soluble protein. The crystal structure of E protein demonstrates that the soluble ectodomain forms a dimer in a head-to-tail orientation (36). We used mouse MAbs that bind to the interface between dimers (40) to determine if E85 expressed from VEE replicons formed dimers. As depicted in Fig. 2, dimer interface MAbs recognized DENV2 E85 protein. These results indicate that at least some, if not all, DENV2 E85 forms dimers. As we have only DENV2 dimer interface antibodies, we could not use this approach to confirm whether DENV1, -3, and -4 also form dimers.

The focus of our study on antibody targeting specificities was EDIII because this region has been identified as a major target of mouse monoclonal antibodies with strong neutralization capacities (41, 43, 82–85). Interestingly, recent studies with human immune sera and monoclonal antibodies indicate that E protein epitopes on EDI and EDII and quaternary epitopes formed by the tight packing of E proteins on the surface of the virus are the main targets of antibodies induced by natural DENV infection of humans (48, 50, 65, 86). Our results here demonstrate that macaques infected with DENV also develop strongly neutralizing antibodies, most of which are not directed against EDIII. Thus, natural infections with DENV in both humans and nonhuman primates seems to lead to antibody responses dominated by non-EDIII-binding antibodies.

In contrast to infection, VRP vaccination with E85 antigen stimulated neutralizing antibodies that mainly targeted epitopes

on EDIII of the vaccine virus serotype only. Our results indicate that monovalent vaccination stimulated EDIII antibodies that bound and neutralized the vaccine serotype, but not the other 3 serotypes. In the tetravalent formulation, the vaccine also appears to stimulate separate populations of type-specific EDIII antibodies that neutralize each serotype. It is unknown why E proteins expressed in the context of a natural DENV infection and VEE replicons expressing E85 stimulate qualitatively different populations of neutralizing antibodies. It has been reported that PrM/M harbors immunodominant epitopes that induce cross-reactive, weakly neutralizing or nonneutralizing antibodies that are not necessary for protection and that may function as decoys to the immune system (46). The absence of prM/M in the E85-VRP vaccine might have helped EDIII become more visible immunologically. In the E85 construct, the EDII fusion loop epitope should be available. We suspect that the E85-VRP vaccine induces a lot of cross-reactive fusion loop antibodies, but it is well documented that these antibodies are weakly neutralizing compared to EDIII antibodies. In fact, we recently reported that removal of all cross-reactive antibodies (which include fusion loop antibodies) from dengue virus immune sera had no effect on the ability of the sera to neutralize dengue virus, indicating that most of the neutralizing antibodies target the type-specific epitopes and not conserved regions, like the fusion loop (49). The similarity in the antibody repertoire after virus infection in NHPs to that in humans suggests that the human antibody response to E85-VRP vaccination could be expected to be, as seen in NHPs, dominated by EDIII specificities and therefore different from that induced by live attenuated virus platforms currently in clinical trials.

The antigen specificities targeted by LAV vaccines have not been reported. One can speculate that LAV-induced specificities may be similar to those induced after natural infection. There is a chance that, due to attenuation, LAV vaccines as a class will not induce enough of the type of immunity required for protection. Therefore, it seems important to accelerate the development and thorough characterization of other vaccine platforms, like the one presented in this paper, as additional approaches. Furthermore, heterologous prime-boost regimes with LAV and E85-VRP vaccines may enhance the response to strongly neutralizing, type-specific EDIII epitopes, which may be underrepresented after a LAV vaccination.

A vaccine with narrow serotype specificity might result in failure of the vaccine to protect against diverse strains within the serotype. This was a concern, since recent studies showed that monoclonal antibodies that bind to epitopes in EDIII may show variable or limited neutralization of other genotypes (37, 42). The E85-VRP vaccine, derived from DENV3 UNC 3001 genotype III, was able to neutralize strains from genotypes I, II, and IV within serotype 3. Although E85-VRP vaccines from serotypes 2, 3, and 4 were not tested independently as monovalent vaccines in this study, we predict that the pattern of inter- and intraserotype neutralizing antibodies will be similar to that seen for DENV3.

After primary infection in humans, cross-reactive antibodies have been implicated in enhancing a secondary infection with a different serotype (87, 88). Waning serotype cross-reactive neutralizing antibodies can facilitate virus entry via the Fcγ receptor and predispose to severe disease via ADE (89). Similarly, priming immunizations with a LAV induced serotype cross-reactive neutralizing antibodies while failing to induce specific antibodies to all four serotypes simultaneously. A further complicating effect of

these cross-reactive neutralizing antibodies is that they interfere with a booster live immunization unless the booster is given 4 to 6 months later (14, 64). The interval between prime and boost thus becomes a period in which vaccinees may be more vulnerable to severe dengue virus than if they did not receive the vaccine in the first place. If a significant proportion of persons who receive the priming dose simply fail to return to the clinic for the booster at 1 year, they may remain vulnerable. We showed that, using the VRP platform, an effective booster immunization could be given after a shorter interval of 6 weeks. The absence of interference by short-lived cross-neutralizing antibodies induced by the VRP prime may be explained by the nonpropagating nature of the VRP and the absence of DENV antigens on the surface of the VRP particle. Therefore, a protective 2-dose vaccine regime may be completed in 6 weeks, reducing the period when the vaccine recipient may be sensitized to severe disease.

Longer-term immunogenicity studies would be needed to address the duration of the neutralizing-antibody response induced by the vaccines beyond 18 weeks after the last immunization. We recognize that establishing a durable immune response to all 4 serotypes may be especially important for a DENV vaccine, and it has been an important goal for dengue vaccine developers. However, because protective efficacy was the primary endpoint of this study, we challenged the monkeys instead of seeking long-term immunity data.

An attempt to compare the potency and efficacy in NHPs of the E85-VRP vaccine with those of LAV candidates currently in clinical trials when they were previously tested in NHPs is described below. The caveats in comparing these studies lie in differences in macaque species, vaccine regimens and schedules, and assays to determine antibody potency and protection. In our study, 16 immunized rhesus macaques showed 100% seroconversion after two immunizations given 6 weeks apart, with Neut_{50} GMTs of 283, 446, 202, and 284 against DENV1 to -4, respectively. Our vaccine induced complete protection against DENV3 and -4 challenges and reduced the duration and peak titer of viremia after challenge with DENV1 and -2. Three LAV vaccines now in clinical trials have been previously tested in HNP. When tetravalent yellow fever-dengue virus chimeric vaccine, ChimeriVax/CYD-TV, was first tested in cynomolgus macaques, a single dose (10^5 PFU of each serotype) resulted in 100% seroconversion, with PRNT_{50} GMTs of 113, 718, 285, and 1,140 against serotypes 1 to 4, respectively, on day 30 postvaccination. All monkeys had neutralization titers at the time of challenge, 6 months later, and 22 of 24 monkeys were protected, as determined by lack of viremia postchallenge (12). A second NHP study (90) was done with serum-free cultured vaccine lot viruses. The study showed interferences among the four CYD serotypes. Immunization at a single anatomical site with TV CYD 5555 (the formulation used in clinical trials) resulted in lower neutralization titers than in the previous study and the need for more than one immunization to get neutralization titers to serotype 2 (90). After two doses given on days 0 and 56, the PRNT_{50} GMTs were 80, <10, 13, and 126 against DENV1 to -4, respectively, in one experiment and 119, <10, <10, and 119 in a second experiment. Out of 8 monkeys, 100% seroconverted to DENV1, 13% to DENV2, 50% to DENV3, and 100% to DENV4. Protective efficacy was not reported in this study. A 1-year booster resulted in 100% seroconversion to DENV1, -3, and -4 and 75% to DENV2 (90). The discrepancies between the two yellow fever-dengue virus chimeric-vaccine studies were attributed to different

culture conditions, animal origins, and assays used to assess viremia and immunogenicity (90). Interestingly, the serotype 2 component of the vaccine was the less immunogenic in macaques (90) and induced no significant protection in the human pilot phase 2b study (19). When this vaccine was tested in flavivirus-naïve humans, the seroconversion rate to three or more serotypes after two doses was ~60%, and 80 to 95% after three doses (18). Based on these results, we feel there is not enough information yet to evaluate the predictive value of the NHP efficacy measurements. When the NIAID's TetraVax-DV vaccine was tested in rhesus macaques, it resulted in 100% seroconversion (defined as 4-fold or greater increase in PRNT after immunization) to all 4 serotypes after a single dose of formulation TV-2, with PRNT_{60} GMTs of 57, 16, 18, and 126 to DENV1 to -4, respectively, measured on day 28 postvaccination (64). Challenge with each DENV serotype homologous strain on day 42 resulted in 100% protection from viremia against challenges with DENV1, -3, and -4, but not -2. Protection against challenge with DENV2 was improved to 100% after a second vaccine dose administered 4 months after the first dose. Importantly, in humans, this vaccine also confers >90% seroconversion to 3 or more serotypes after 1 dose (A. Durbin, presented at the American Society of Tropical Medicine and Hygiene 61st annual meeting, Atlanta, GA, 2012). The Inviragen tetravalent LAV, DENVax formulation 2 (3355), when tested in cynomolgus macaques induced seroconversion rates to each serotype of 87.5%, 100%, 100%, and 100% after 2 doses, with PRNT_{50} GMTs of 306, 554, 320, and 36 (91). Based on these earlier reports and the results presented here, we anticipate that when tested in humans, the TV E85-VRP vaccine will induce serotype-specific neutralizing antibodies to all 4 DENVs after 2 doses while inducing a qualitatively different antibody response with enhanced EDIII specificity compared to that of LAV vaccines.

We were unable to establish a correlation between the prechallenge titer and protection from viremia. There were 2 macaques in the DENV1 challenge group and 3 in the DENV2 challenge group that had detectable viremia, although reduced in magnitude and duration. Prechallenge Neut_{50} titers in those animals were 23, 112, 174, 170, and 50, which were not different from those in other macaques with no detectable viremia by focus-forming assay. These results suggest that neutralizing antibodies as measured in this study are not the only determinants of protection in this model.

There was a robust anamnestic response after challenge in the VRP-vaccinated animals. The priming afforded by the vaccine indicates that it is sufficient not only to reduce replication of the challenge virus significantly, but also to prime a strong anamnestic response consistent with long-lived immunity. Therefore, in an endemic setting, vaccination combined with subsequent subclinical infection could result in particularly strong and long-lasting protective immunity.

A tetravalent E85-VRP vaccine was successful in overcoming serotype dominance/interference when the four components of the vaccine were mixed. One vaccine dose was sufficient to induce a tetravalent response in 56% of nonhuman primates, and 100% seroconversion was achieved after 2 doses. After adjustment of the ratio of monovalent vaccines in the combination, there were no signs of dominance or viral interference among the 4 VRP vaccine components. First, 100% seroconversion to all 4 serotypes was achieved after a two-dose regime with a 6-week interval, which duplicated the rate of seroconversion for the monovalent vac-

cines. Second, the mean Neut₅₀ titer against DENV3 (GMT = 202) elicited by the tetravalent formulation (Table 3) was not significantly diminished relative to the mean titer against DENV3 elicited by the monovalent DENV3 E85-VRP (GMT = 214) (Table 1) given at the same dose, suggesting that the other components of the tetravalent VRP mixture did not interfere with the DENV3 VRP component. Although here we compared only the monovalent versus tetravalent responses for the serotype 3 component, in mice we compared each vaccine component independently and in the tetravalent mixture and showed for each serotype that the response in the tetravalent mixture was as high as that in the monovalent formulation (White, unpublished).

Although we did not test the tetravalent vaccine sera for neutralization against multiple members of each serotype, the DENV strains used in the neutralization assay were heterologous WHO reference strains and therefore measured a broader neutralization response.

The relative doses of the 4 components of the tetravalent E85-VRP vaccine were 1:1:1:2, for serotypes 1, 2, 3, and 4, respectively. This ratio was based on mouse studies in which E85 from DENV4 was less immunogenic than the other serotypes when equal doses were administered as a tetravalent VRP cocktail (White, unpublished). Increasing the dose of DENV4 E85-VRP 2-fold relative to the other serotypes resulted in DENV4 neutralizing-antibody titers that were similar to those of the other 3 serotypes after the first or second immunization. This suggests that the immune responses of individual serotypes may be modulated successfully by changing the ratio of the VRP vaccine components.

The VRP platform combines advantages of both LAV and killed/subunit vaccines. Similar to LAV, E85-VRP contains a replicating viral RNA capable of inducing all arms of the immune system, including a strong innate immune response. On the other hand, as in killed and subunit vaccines, the components of the tetravalent mixture are nonpropagating antigens; therefore, interference is minimized, resulting in a more balanced immune response early after immunization.

The work presented here demonstrates in nonhuman primates that a single-cycle alphavirus vector expressing a truncated soluble form of the major DENV envelope protein is a promising second-generation tetravalent vaccine candidate. As the E85-VRP tetravalent candidate exhibits the major characteristics of a potentially successful dengue vaccine, we suggest that further testing in clinical trials is justified, including heterologous prime-boost regimes with live attenuated vaccine candidates.

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