Immunogenicity and Efficacy of Flagellin-Envelope Fusion Dengue Vaccines in Mice and Monkeys

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The envelope (E) protein of flaviviruses includes three domains, EI, EII, and EIII, and is the major protective antigen. Because EIII is rich in type-specific and subcomplex-specific neutralizing epitopes and is easy to express, it is particularly attractive as a recombinant vaccine antigen. VaxInnate has developed a vaccine platform that genetically links vaccine antigens to bacterial flagellin, a Toll-like receptor 5 ligand. Here we report that tetravalent dengue vaccines (TDVs) consisting of four constructs, each containing two copies of EIII fused to flagellin (R3.2x format), elicited robust and long-lived neutralizing antibodies (geometric mean titers of 200 to 3,000), as measured with a 50% focus reduction neutralization test (FRNT50). In an immunogenicity study, rhesus macaques (n = 2) immunized subcutaneously with 10 µg or 90 µg of TDV three or four times, at 4- to 6-week intervals, developed neutralizing antibodies to four dengue virus (DENV) serotypes (mean post-dose 3 FRNT50 titers of 102 to 601). In an efficacy study, rhesus macaques (n = 4) were immunized intramuscularly with 16 µg or 48 µg of TDV or a placebo control three times, at 1-month intervals. The animals that received 48-µg doses of TDV developed neutralizing antibodies against the four serotypes (geometric mean titers of 49 to 258) and exhibited reduced viremia after DENV-2 challenge, with a group mean viremia duration of 1.25 days and 2 of 4 animals being completely protected, compared to the placebo-treated animals, which all developed viremia, with a mean duration of 4 days. In conclusion, flagellin-EIII fusion vaccines are immunogenic and partially protective in a nonhuman primate model.

Dengue disease poses a significant health threat to almost one-half of the world’s population residing in the Asian-Pacific region and Latin America, where the disease is endemic, as well as to travelers and military personnel. Currently, no licensed dengue vaccine is available. Several vaccine candidates based on live attenuated dengue viruses (DENVs) or dengue virus-flavivirus chimeras are currently being evaluated in phase II or III clinical trials (for reviews, see references 1–3). However, some of these vaccines require lengthy immunization regimens of up to 1 year for the development of immunity to all four serotypes, which seems to make them less well suited for travelers and military personnel. In a series of recently published phase Ib/II trials conducted in five Asian countries in which dengue is endemic, the Sanofi-Aventis chimeric yellow fever (YF) 17D-DENV-1 to -4 CYD tetravalent dengue vaccine (TDV) showed an acceptable safety profile and 56% overall efficacy (4); however, the vaccine failed to confer significant protection against DENV-2 (5). These findings underscore the need for the development of alternative vaccine platforms, such as those based on purified inactivated virus (6), DNA (7), and recombinant subunit truncated envelope (E) proteins (e.g., 80% envelope protein [80E]) (8). Some of these alternative vaccine candidates have been evaluated in phase I trials, where they were found to be safe and immunogenic while allowing for much shorter immunization regimens.

The dengue virion E protein, which functions in receptor binding and membrane fusion, is the major protective antigen of flaviviruses, containing most of the virus-neutralizing epitopes (9, 10). The E ectodomain, which constitutes approximately 80% of the protein (80E), contains three distinct domains that have been identified immunologically (11, 12) and by X-ray crystallography (13). E domain I (EI) is the central domain, and E domain II (EII) is the dimerization domain and contains the flavivirus conserved fusion peptide. The carboxyl-terminal E domain III (EIII) is the smallest domain (approximately 100 amino acids in length) and contains the putative receptor binding site as well as type-specific and subcomplex-specific neutralizing epitopes that are recognized by strongly neutralizing antibodies (11, 14–17), making it an attractive target for subunit vaccine development. However, EIII fusion proteins delivered by plasmid DNA or purified from Escherichia coli or baculovirus expression systems are often poorly immunogenic, requiring relatively high antigen doses or potentially toxic adjuvants to achieve moderate immunogenicity and protective efficacy in animal models (18).

VaxInnate has developed a clinically proven recombinant flagellin-antigen fusion platform to allow rapid development and economical manufacturing of fusion protein–based vaccines using a well-established E. coli fermentation system and a standardized purification process. This vaccine platform contains bacterial...
flagellin (a Toll-like receptor 5 [TLR5] ligand) genetically fused to the antigen of choice at a number of possible linkage sites (Fig. 1A to C), eliciting robust neutralizing antibody responses by linking innate immunity and adaptive immunity. Candidate influenza vaccines based on this platform, in which the globular head of influenza hemagglutinin (HA) was fused to Salmonella enterica serovar Typhimurium FljB flagellin phase 2 (STF2) at the carboxyl terminus, replacing domain 3 (R3), or at both the C-terminal and R3 positions (R3.2x), were demonstrated to be immunogenic and efficacious in mice (19–21) and ferrets (22). More importantly, these vaccine candidates are well tolerated and immunogenic in humans (23, 24). Among the three vaccine formats, R3.2x is the most immunogenic and has the widest safety window (25). The VaxInnate first-generation flavivirus vaccines based on flagellin-EIII fusion proteins have been demonstrated to elicit protective immunity to West Nile virus (WNV) (26), Japanese encephalitis virus (JEV), and DENV-2 (W. F. McDonald, J. W. Huleatt, H. G. Foellmer, D. Hewitt, J. Tang, P. Desai, A. Price, A. Jacobs, V. N. Takahashi, Y. Huang, V. Nakaar, and T. J. Powell, unpublished results) in mice. Here we report studies to evaluate the immunogenicity and efficacy of tetravalent dengue vaccine (TDV) candidates in mice and in a nonhuman primate (NHP) model.

**MATERIALS AND METHODS**

**Cells and viruses.** African green monkey kidney epithelial cells (Vero) were obtained from ATCC (Manassas, VA) and maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). DENV-1 (strain West Pac 74), DENV-2 (strain S16803), DENV-3 (strain CH53489), and DENV-4 (strain TVP360) were kindly provided by Robert Putnak (Walter Reed Army Institute of Research [WRAIR], Silver Spring, MD). The virus stocks were amplified by infection of Vero cells in MEM, supplemented with 2% FBS and antibiotics, for 5 to 7 days at 37°C. Aliquots of harvested virus were stored at −80°C until use. Virus stocks were titrated with standard plaque assays.

**Cloning and expression system.** Codon-optimized synthetic genes encoding the EIII domain (amino acids 289 to 400) of DENV-1 (strain 16007), DENV-2 (strain 16681), DENV-3 (strain Nicaragua 24/94), or DENV-4 (strain 341750) for E. coli expression were obtained from a commercial vendor (Integrated DNA Technologies, Coralville, IA). For each strain, two EIII domain copies were fused to flagellin (STF2). One copy replaced the D3 domain of STF2, and the other copy was fused to the C terminus of STF2. The final DNA construct was cloned into the pET24a vector, and the sequence was confirmed by an outside vendor (Genewiz Inc., South Plainfield, NJ). The plasmid was transformed into E. coli BLR(DE3) (Novagen) for protein expression.

**Protein purification.** Following fermentation and isopropyl-β-D-thiogalactopyranoside (IPTG) induction, the flagellin fusion proteins were purified from E. coli cell lysates using Ni-NTA agarose (Qiagen) and buffers containing 150 to 300 mM imidazole. Purified proteins were characterized by SDS-PAGE and Western blotting.

**FIG 1** (A, B, and C) Domain structures and alternative formats of flagellin fusion vaccines. For illustrative purposes, R3 (A), C-terminal (C-term) (B), and R3.2x (C) vaccine formats are shown fused to an influenza HA antigen (green). Flagellin domains (D0, D1, D2, and D3) are labeled. The R3.2x vaccine contains 2 copies of the same antigen; one copy is fused to the C terminus of flagellin and the other replaces domain 3. (D) SDS-PAGE analysis of R3.2x EIII dengue vaccine candidates. Purified proteins (2.5 μg/lane) were separated on a 4 to 12% SDS-PAGE gel and stained with Coomassie blue. Lane 1, R3.2xD1EIII; lane 2, R3.2xD2EIII; lane 3, R3.2xD3EIII; lane 4, R3.2xD4EIII; lane M, protein markers (in kDa). (E and F) Replicate gels were transferred to Immobilon membranes for Western blot analyses and were probed with rabbit polyclonal antiflagellin serum (E) or the DENV-2-specific MAb 3H5 (F) and then with HRP-conjugated goat anti-rabbit IgG or goat anti-mouse IgG (each at 1:1,000 dilution). The antibody-specific bands were visualized with a Pierce CN (4-chloro-1-naphthol)/DAB (3,3′-diaminobenzidine) substrate kit.
were recovered from inclusion bodies and purified using chromatography involving cell disruption and clarification, denaturation, refolding, and capture (26). An additional polishing step with a ceramic hydroxyapatite (CHT) gel filtration step was necessary for purification of proteins in the R3.2x format. The proteins were formulated in PBS (10 mM sodium phosphate, 137 mM NaCl, 2.7 mM KCl [pH 7.4]). Purified fusion proteins were analyzed by SDS-PAGE and detected as protein bands with the predicted molecular sizes. Endotoxin levels (measured with a Limulus amoebocyte lysate [LAL] test) for all lots used in this study were ≤40 endotoxin units (EU)/mg. Finally, all vaccine candidates induced significant dose-dependent interleukin 8 (IL-8) production in an *in vitro* TLR5 assay using HEK293 cells, as described previously (20). The baculovirus-expressed 80E proteins of all four serotypes were purified from culture medium with affinity chromatography, according to the manufacturer’s instructions (Invitrogen).

**Measurement of monoclonal antibody binding with enzyme-linked immunosorbent assay.** Binding of the DENV-2 vaccine candidate (R3.2xD2EIII) to the type-specific monoclonal antibody (Mab) 3H5 (ATCC HB-46) was measured with an enzyme-linked immunosorbent assay (ELISA), as described previously (26). The neutralizing Mab 3H5 recognizes an epitope in DENV-2 E III (11, 14). Serially diluted R3.2xD2EIII and DENV-2 80E in duplicate were coated on 96-well ELISA plates. After overnight incubation at 4°C and blocking with assay diluent (SuperBlock buffer for 2 to 3 h at room temperature and washed with PBS-T), the plates were incubated for 1.5 h, and subsequently incubated with 1% Ultra TMB substrate, the reaction was stopped with 1 M H2SO4 and the optical density at 450 nm (OD450) was measured with a microplate spectrophotometer. Data were analyzed using a 4-parameter-fit curve format (R3.HA1) was included as a positive control. Three hours later, with control or test vaccines at 1 μg each. An influenza vaccine in R3 format (R3.HA1) was included as a positive control. Three hours later, blood was collected. Sera were prepared and serum levels of proinflammatory cytokines were measured with a cytometric bead array (CBA) mouse inflammation kit (BD Biosciences), following the manufacturer’s instructions. Cytokine concentrations were extrapolated from standard curves with detection limits of 5.0 and 7.3 pg/ml for IL-6 and tumor necrosis factor alpha (TNF-α), respectively.

**Measurement of serum antibodies.** Envelope-specific IgG was measured by ELISA as described previously (21, 26). Briefly, ELISA plates were coated overnight at 4°C with baculovirus-produced DENV-2 80E protein (0.3 μg/well) in PBS. Purified IgG standard (AbD Serotec) of monkey origin was serially diluted and similarly coated. The plates were blocked with SuperBlock buffer for 2 to 3 h at room temperature and washed with PBS-T. Serially diluted sera were added, the plates were incubated for 1.5 h and washed, and detection was performed with HRP-conjugated goat anti-mouse IgG. All washes between reagent addition steps were performed 3 times with PBS-T. After monitoring of color development with Ultra TMB substrate, the reaction was stopped with 1 M H2SO4 and the OD450 was measured with a microplate spectrophotometer. Serum levels of IgG were extrapolated from an IgG standard curve and expressed as geometric means and 95% confidence intervals (CIs).

**Immunogenicity studies in mice.** Mouse studies were conducted at Princeton University (Princeton, NJ), under a protocol approved by the institutional animal care and use committee (IACUC) and in accordance with NIH guidelines (36). The Princeton facility is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) and holds U.S. Public Health Service (PHS) assurance (assurance A3434-01). Female BALB/c mice (6 to 8 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA) and maintained at the AAALAC-accredited animal facility of Princeton University. Vaccines were formulated in FI47 formulation buffer (10 mM Tris, 10 mM histidine, 150 mM NaCl, 5% trehalose, 0.02% Polysorbate 80, 0.1 mM EDTA, 0.5% ethanol [pH 7.0]) prior to animal studies. Groups of 8 to 10 mice were immunized subcutaneously (s.c.) on days 0, 21, and 42 and were bled on day 35 (after dose 2) or day 56 (after dose 3). Serum samples were stored at −70°C until use.

**Immunogenicity and efficacy studies in rhesus macaques (Macaca mulatta).** Two monkey studies were conducted, at the University of Texas Medical Branch (UTMB) and the Walter Reed Army Institute of Research (WRAIR), under IACUC-approved protocols. Both protocols were also approved by the U.S. Army Medical Research and Materiel Command (USAMRMC) Animal Care and Use Review Office (ACURO). The UTMB and WRAIR comply with all applicable U.S. federal regulations, including the Animal Welfare Act (U.S. Department of Agriculture [USDA]), the U.S. PHS policy on humane care and use of laboratory animals (NIH), the U.S. government principles for the utilization and care of vertebrate animals used in research, teaching, and testing, developed by the Interagency Research Animal Committee (IRAC), and other federal statutes and state regulations related to animal research. The animal care and use program conducts reviews involving animals in accordance with NIH guidelines (36).

The first nonhuman primate study was conducted at the UTMB to evaluate the immunogenicity of two tetravalent vaccine formulations. Groups of two flavivirus-negative male rhesus macaques (3 to 5 kg; Charles River Laboratories) were anesthetized with ketamine (10 mg/kg, administered intramuscularly [i.m.]) and immunized s.c. with 5 or 10 μg TDV10 (1.5, 0.5, 1.5, and 6.5 μg of R3.2xD1EIII, R3.2xD2EIII, R3.2xD3EIII, and R3.2xD4EIII, respectively) or 90 μg (TDV90) (13.5, 4.5, 13.5, and 58.5 μg of R3.2xD1EIII, R3.2xD2EIII, R3.2xD3EIII, and R3.2xD4EIII, respectively) of tetravalent dengue vaccines on days 0, 28, 56, and 98. Blood was collected using 10-ml Vacutainer SST venous blood collection tubes (BD) on the day of each immunization and 2 and 4 weeks after each immunization. Serum samples were prepared following centrifugation and were stored at −70°C until use.

In the second NHP study, the immunogenicity and efficacy of two TDV formulations were evaluated using an established DENV-2/rhesus macaque infection model, at the WRAIR. Groups of four flavivirus-negative rhesus macaques (3 to 5 kg; Covance, Alice, TX) were anesthetized with i.m. injection of a mixture of ketamine (11 mg/kg) and acetylmorphone (0.53 mg/kg) and were immunized i.m. with TDV, at a high (48 μg) or low (16 μg) dose, on days 0, 30, and 60. On day 91, the monkeys were challenged s.c. with DENV-2 (strain S16803; 107 PFU/animal in 0.5 ml PBS). Blood was collected using Vacutainer SST venous serum separator tubes.
(BD) on the day of each immunization and 2 and 4 weeks after each immunization (5 ml each time), as well as daily on days 91 to 103 (2 ml/day). Serum samples were prepared following centrifugation and were stored at -70°C until use.

**Viremia assays.** Serum samples collected daily for 14 days postchallenge were aliquoted and frozen at -80°C prior to testing. Virus-positive serum samples were identified using an amplified viremia assay (limit of detection, ~10 PFU/ml), as follows. Sera (0.1 ml) were inoculated onto newly confluent Vero cell monolayers in 25-cm² (T-25) flasks. Following inoculation, cultures were incubated at 35°C for 1 h (to allow for virus adsorption), fed with MEM containing 2% heat-inactivated FBS, and incubated at 35°C, with a complete medium replacement on day 7. Virus was detected by plaque assays of the 14-day culture supernatants on confluent Vero cell monolayers in 6-well plates and staining with neutral red. Samples that exhibited any virus plaques were scored as positive. Virus titers were then determined for each positive serum sample by direct plaque assays of a fresh serum aliquot at dilutions of 1:10, 1:100, and 1:1,000 on Vero cells in 6-well plates (duplicate wells per dilution). Following staining with neutral red, the plaques were counted and the average number of plaques at each serum dilution was used to calculate a virus infectivity titer, expressed as PFU per ml of serum.

**Statistical analyses.** For immunogenicity analyses, FRNT₅₀ titers were logarithmically transformed and analysis of variance (ANOVA) and Tukey’s multiple-comparison tests were performed to determine whether there were significant differences in immunogenicity between the groups. Statistical analyses of viremia occurrence and mean days of viremia over the indicated period were performed using Fisher’s exact test and the Mann-Whitney test, respectively, at a significance level of <0.05, in GraphPad Prism 5.0 (San Diego, CA).

**RESULTS**

VaxInnate has developed several formats (C term, R3, and R3.2x) of flagellin-EIII dengue vaccines (Fig. 1A to C). R3.2x was found to be the most immunogenic form, with the widest safety window, for a flagellin-HA influenza vaccine in humans. In our initial comparative mouse studies of dengue vaccines, R3.2x also appeared to be the most suitable format for the development of tetravalent formulations (data not shown). Therefore, our further work focused on the development of dengue vaccines in the R3.2x format. The fusion proteins were successfully expressed and purified to homogeneity (~90% purity) as 67.2-kDa polypeptides (major bands) on SDS-PAGE (Fig. 1D). The major protein bands reacted with both rabbit antiflagellin antiserum (Fig. 1E) and DENV-2 EIII-specific MAb 3H5 (Fig. 1F) in Western blots. The minor bands below the target proteins are thought to be truncated fusion proteins, as the proteins reacted with rabbit antiflagellin antiserum (Fig. 1E) but not 3H5 (Fig. 1F).

We next compared the 3H5-binding activities of the DENV-2 vaccine candidate (R3.2xD2EIII) and baculovirus-expressed DENV-2 80E, which consists of domains E, EI, and EII. The ELISA results showed lower levels of binding of R3.2xD2EIII to envelope-specific MAb 3H5, compared with that of DENV-2 80E (Fig. 2A). The midpoint value of the R3.2xD2EIII curve (3.88 nM) was 1/3.8 that of the DENV-2 80E curve (1.03 nM).

We developed an in vivo TLR5 assay during the development of flagellin-HA fusion vaccines (27). TLR5 activity was assessed by quantifying innate immunity cytokine levels stimulated by vaccine candidates in mice. Three hours following immunization, significant levels of IL-6 and TNF-α were elicited with 1 μg of DENV-1 and DENV-3 vaccine candidates (Fig. 2B and C). We regard the levels of cytokine induction as indicating moderate TLR5 activity, compared with the influenza vaccine in the R3 format (R3.HA1 or VAX128B), which was shown to be highly immunogenic in humans (25).

We next evaluated the immunogenicity of a candidate TDV formulation in mice. Groups of 10 BALB/c mice were immunized s.c. on days 0, 21, and 42 with a TDV consisting of 2.5 μg R3.2xD1EIII, 1 μg R3.2xD2EIII, 2.5 μg R3.2xD3EIII, and 10 μg R3.2xD4EIII, and sera were obtained on days 35 and 56 for measurement of virus-neutralizing antibodies. Following two immunizations, mice developed FRNT₅₀ antibody geometric mean titers (GMTs) of 557, 368, 320, and 106 for DENV-1, DENV-2, DENV-3, and DENV-4, respectively (Fig. 3A); after three immunizations, the GMTs increased to 1,280, 2,560, 485, and 243, respectively (Fig. 3A). These results demonstrate that 2 or 3 immunizations with the TDV elicited robust, high-titer, neutralizing

**FIG 2** Antigenicity and TLR5 activity of flagellin-EIII fusion antigens. (A) Binding of vaccine candidate R3.2xD2EIII to DENV-2 envelope-specific MAb 3H5. ELISA plates were coated with 1:2 serially diluted R3.2xD2EIII or DENV-2 80E in PBS and reacted with 3H5, and detection was performed with HRP-conjugated goat anti-mouse IgG. The mean OD₄₅₀ values of duplicates were read and graphed. (B and C) In vivo TLR5 activity, BALB/c mice (n = 5) were immunized with 1 μg of R3.2x EII vaccine candidates. An influenza vaccine in the R3 format (R3.HA1) was included as a positive control. Three hours later, serum samples were collected. Serum levels of IL-6 (B) and TNF-α (C) were measured with a BD CBA kit and are expressed as geometric means (longer horizontal lines) and 95% CIs (shorter horizontal lines). **, P < 0.01 versus the F147 group in Mann-Whitney test.
antibody responses, particularly for DENV-1 and DENV-2, with somewhat lower titers for DENV-3 and DENV-4.

One of the major concerns for the development of EIII and other subunit dengue vaccines is the lack of durable antibody responses (18, 31). We determined the longevity of the neutralizing antibody responses in mice for a TDV candidate consisting of R3.2xD1EIII, R3.2xD2EIII, R3.2xD3EIII, and R3.2xD4EIII. Mice were immunized s.c. three times, at 3-week intervals, and sera were collected over a 7-month period following dose 3 for the measurement of virus-neutralizing antibody responses to DENV-1 to -4. The results demonstrated that the TDV vaccine in the R3.2x format elicited stable neutralizing antibody titers for DENV-2, DENV-3, and DENV-4 that lasted at least 7 months; although the neutralizing titers for DENV-1 declined approximately 8-fold over the same period, they persisted at a level greater than 200 (Fig. 3B). Overall, the flagellin-EIII fusion vaccine candidate elicited robust and durable neutralizing antibody responses in mice.

We next evaluated the immunogenicity of the TDV candidates in a NHP model. In a small pilot study, groups of 2 rhesus macaques per group were immunized s.c. on days 0, 28, 56, and 98 with TDVs at a total dose of 10 μg (TDV10) (1.5 μg for DENV-1, 0.5 μg for DENV-2, 1.5 μg for DENV-3, and 6.5 μg for DENV-4) or 90 μg (TDV90) (13.5, 4.5, 13.5, and 58.5 μg, respectively). Sera were collected on days 0, 28, 56, 70, 84, 98, 112, and 126 for measurement of DENV-neutralizing and 80E-specific IgG antibody responses. Following three immunizations with the TDVs, all animals seroconverted (FRNT50 titers of 10) with DENV-neutralizing antibodies for all four DENV serotypes, with the highest antibody titers for DENV-1 and DENV-3 (Fig. 4).

In terms of durability of the immune responses to the TDVs, DENV-neutralizing antibody titers peaked at week 18 after primary immunization and then declined approximately 3- to 7-fold,
although all animals remained seropositive at the final time point (Fig. 4). We also measured antigen (80E)-specific IgG levels of immune sera, as Simmons et al. demonstrated that total antibody levels and antibody avidity measured by ELISA correlated with virus-neutralizing antibody titers and with protection of NHPs against viremia (31). Envelope-specific IgG titers peaked on day 98, at the time of the fourth immunization, and then declined approximately 6-fold and 10-fold in the TDV90 and TDV10 groups, respectively, during the 4-month follow-up period (data not shown).

We then assessed the immunogenicity and efficacy of two TDV formulations (low and high doses) consisting of 4 monovalent R3.2x EIII fusion proteins in a DENV-2/NHP challenge model (Table 1). The results demonstrated that the high-dose TDV formulation (48 μg in total) elicited moderate neutralizing antibody titers for three (DENV-2, DENV-3, and DENV-4) of the four dengue serotypes following three i.m. immunizations (Fig. 5). Among the 4 serotypes, the TDV formulations elicited the highest geometric mean titers for DENV-2, followed by DENV-4, DENV-3, and DENV-1. The results also demonstrated that the TDV formulations elicited envelope antigen-specific IgG responses in the vaccinated animals in a dose-dependent manner, with peak antibody titers being achieved at day 60 (after dose 2) and day 90 (after dose 3) (Fig. 6).

In order to determine vaccine efficacy, animals in the vaccine and control groups were challenged 1 month after dose 3 with the S16803 strain of DENV-2 (10⁵ PFU, administered s.c.), and viremia was measured in sera collected daily after the challenge, with a qualitative virus amplification assay and a quantitative direct plaque assay in Vero cell cultures. The results demonstrated that low-dose TDV (16 μg in total) failed to significantly reduce viremia in the vaccinated animals (Table 2). In contrast, two of the four monkeys immunized with the high-dose TDV (48 μg in total) were free of viremia (50% protection). The frequency of viremic days over the total number of days was significantly lower in the TDV48 group than in the placebo group (P = 0.005). The mean days of viremia in the high-dose group were also lower than the value for the placebo group (P = 0.055). Although mean peak titers were lower in the vaccine groups (73 PFU/ml for the TDV16 group and 6.3 PFU/ml for the TDV48 group) than in the placebo group (105 PFU/ml), the reduction was not statistically significant. Interestingly, there was no apparent correlation between viremia and prechallenge FRNT₅₀ titers for individual animals. For example, one animal (animal 09U004) in the TDV48 group showed the lowest FRNT₅₀ titer but was viremia free. In contrast, another animal in the same group exhibited a higher antibody titer but showed breakthrough viremia for 2 days. Overall, the results demonstrated that the TDV candidate, as tested, was immuno-

**TABLE 1 Design for study of immunogenicity and efficacy of TDVs in NHPs**

| Group (immunization amounts)ᵃ | No. of monkeys/group | Immunization days | Bleeding days | Challenge day |
|------------------------------|----------------------|------------------|---------------|--------------|
| TDV48 (9, 9, 6, and 24 μg)   | 4                    | 0, 30, 60        | 0, 30, 60, 90-103, 120 | 91           |
| TDV16 (3, 3, 2, and 8 μg)   | 4                    | 0, 30, 60        | 0, 30, 60, 90-103, 120 | 91           |
| Placebo (F147 buffer)       | 4                    | 0, 30, 60        | 0, 30, 60, 90-103, 120 | 91           |

ᵃ Numbers in parentheses indicate doses of R3.2xD1EIII, R3.2xD2EIII, R3.2xD3EIII, and R3.2xD4EIII, respectively.

**FIG 5 Neutralizing antibody titers elicited by TDVs.** Rhesus monkeys (3 to 5 kg; n = 4) were immunized three times and bled as outlined in Table 1. Neutralizing antibody titers of post-dose 2 and post-dose 3 sera were measured by FRNT₅₀ and are expressed as geometric mean titers (GMTs).
DISCUSSION

These studies demonstrated that a tetravalent dengue vaccine (TDV) consisting of 4 monovalent flagellin-EIII components in the R3.2x format was immunogenic in mice and a NHP model following a relatively short immunization regimen. In this study, we show that the EIII-specific neutralizing MAb (3H5) binding of the DENV-2 candidate was somewhat lower than that of DENV-2 80E. This is consistent with the Western blot results, which indicated that a small proportion of the R3.2xD2EIII fusion protein (truncated fusion protein as a minor band) lost binding activity for the EIII-specific neutralizing MAb 3H5. The small antigen size could render EIII more susceptible to degradation during purification. This is also consistent with our previous findings with flagellin-WNV EIII antigen, which demonstrated that one of five conformationally sensitive epitopes on the fusion protein was not well preserved (26).

The antibody responses to vaccination were robust and durable in mice but only moderate and relatively short-lived in rhesus macaques. These different antibody responses could be explained by recent findings that suggest that EIII epitopes are immunodominant in mice (14, 16), whereas humoral immune responses in humans and NHPs following dengue infection appear to target mainly the epitopes surrounding EII and the EI-EII hinge region (32, 33). However, White et al. demonstrated that a nonreplicating alphavirus replicon particle (VRP) expressing soluble E dimer (E85-VRP) elicited protective immunity in NHP infection models (34). More importantly, E85-VRP induced only type-specific antibodies predominantly targeting EIII (34). This feature is different from findings for live/chimeric virus-based or prME-VRP-based vaccines and indicates that EIII-targeting antibodies can be protective in a NHP model. The short-lived antibody responses could be partly due to suboptimal helper T cell functions associated with the relatively small EIII antigen (~100 amino acids), compared to 80E (~400 residues).

We observed partial protection from viremia (used as a surrogate indicator of disease) in the DENV-2/NHP model only in the high-dose vaccine group, as determined by the number of viremia-free animals and a reduction in the group mean days of viremia, compared to placebo controls. Similar to other NHP studies, one limitation of this work was the small group sizes (n = 2 to 4), which limited the power of statistical analyses. Overall, our results with the flagellin-EIII fusion candidates are consistent with previously published results obtained with high doses (50 to 500 μg) of EIII fusion proteins formulated in an exogenous adjuvant (31, 35, 36). Utilizing a similar DENV-2/NHP model, Simmons et al. observed relatively poor durability of antibody responses and lack of protection from viremia in rhesus macaques (n = 4) following three immunizations (days 0, 30, and 60) with Freund’s adjuvanted EIII (amino acids 298 to 400)-maltose-binding pro-
tein (MBP) antigen (500 μg) and challenge (5 months after the third dose) with the same strain of DENV-2 used to produce the recombinant antigen (31). Other laboratories observed variable protection (ranging from about 50% to 100%) of NHPs (2 or 3 animals per group) following four immunizations with Freund’s adjuvanted DENV-1 or DENV-2 EIII fused to the P64k protein of Neisseria meningitidis (35, 36). It is noteworthy that these chimeric antigens included a longer EIII sequence (amino acids 286 to 426) than was used for our studies, suggesting that additional C-terminal residues may contribute to protective immunogenicity of DENV EIII. Additionally, four immunizations were given with longer intervals (days 0, 30, 90 or 94, and 210 or 150) in those studies. Results from our group and others suggest that booster immunizations may be required to maintain antibody levels, consistent with the previous report of another recombinant EIII fusion vaccine candidate (31).

We observed different kinetics of antigen (80E)-binding and virus-neutralizing antibody responses in this study. Whereas 80E-binding IgG levels peaked after 2 immunizations, the virus-neutralizing antibody titers increased after the third immunization (Fig. 5 and 6). Neutralizing antibodies are thought to account for a part of total antigen-binding IgG amounts. This could reflect a better B cell maturation event that might have led to greater avidity of antigen-specific antibodies and/or greater proportions of neutralizing antibodies.

It is important to note that the adjuvant effect of flagellin in the fusion proteins (9 μg of the DENV-2 component) was clearly evident in our studies. We think that the adjuvant effect is mainly mediated via the TLR5 signaling pathway. We demonstrated that R3.2x EIII candidates stimulated innate immunity cytokines (TNF-α and IL-6) in this study (Fig. 2B and C). This is consistent with our previous report that a flagellin-WNV EIII fusion protein stimulated TLR5 in RAW264.7 cells expressing human TLR5, as measured by TNF-α induction (26). The West Nile virus vaccine candidate based on the flagellin-EIII fusion protein elicited significantly lower antigen-specific IgG levels in TLR5-knockout mice than in wild-type mice (26). The core of the platform is the fusion of the genetic sequence for a TLR5 ligand to the genetic sequence for the vaccine antigen, as codelivery of EIII and flagellin resulted in low antibody responses in mice (26). TLRs are expressed on various cell types, particularly professional antigen-presenting cells (APCs), where they act as primary sensors of microbial products and activate signaling pathways that lead to the induction of immune responses (37–40). Influenza vaccines based on flagellin-HA fusion proteins were shown to be well tolerated and immunogenic in over 1,100 human subjects (23–25). Neutralizing antibodies were elicited against the 4 serotypes of DENV in both NHP studies. However, neutralizing antibody titers elicited via the s.c. route using different dose ratios in the first study were higher than those elicited via the i.m. route in the second study. Alternative routes of administration or dosing intervals could improve titers and antibody longevity; however, the suboptimal efficacy could have resulted from insufficient antigenic sites (neutralizing epitopes) within the EIII domain. Consistent with this hypothesis, recent findings indicate that EIII-specific antibodies account for a small fraction of the virus-neutralizing antibodies following DENV infections in humans (32, 33, 41, 42) and monkeys (34), which may support the idea that EIII is not as immunodominant in primates as it is in mice. Additionally, recombinant 80E proteins, which contain all three domains, have been shown to be highly immunogenic and efficacious in NHP models when formulated in Iscomatrix, a saponin-based adjuvant (43, 44). Thus, we hypothesized that limited or compromised presentation of neutralizing epitopes afforded by the EIII antigens, plus an absence of specific T helper epitopes that may be present in the E1 and EII domains, might have rendered EIII-based vaccines less effective in inducing long-lasting neutralizing antibody responses. A recent study of human monoclonal antibodies following natural dengue infections led to identification of the envelope dimer epitope against which monoclonal antibodies are cross-reactive and broadly neutralizing in vitro (45). Although this finding has important implications for vaccine design, it is worth noting that immune responses after immunization can be different from those following natural dengue infections. In fact, a subunit dengue vaccine based on adjuvanted monomeric 80E is highly immunogenic and efficacious in the NHP model (43, 44). Further, a tetravalent dengue vaccine based on Iscomatrix-adjuvanted 80Es was well tolerated and immunogenic in a phase I clinical study (46). The results from our ongoing studies and those of other investigators suggest that an ideal subunit dengue vaccine antigen should include all three domains of the envelope protein or at least some additional important epitopes outside the EIII region. Therefore, our current efforts are focused on developing flagellin-80E fusion proteins.

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