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Construction, Safety, and Immunogenicity in Nonhuman Primates of a Chimeric Yellow Fever-Dengue Virus Tetravalent Vaccine

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We previously reported construction of a chimeric yellow fever-dengue type 2 virus (YF/DEN2) and determined its safety and protective efficacy in rhesus monkeys (F. Guirakhoo et al., J. Virol. 74:5477–5485, 2000). In this paper, we describe construction of three additional YF/DEN chimeras using premembrane (prM) and envelope (E) genes of wild-type (WT) clinical isolates: DEN1 (strain PUO359, isolated in 1980 in Thailand), DEN3 (strain PaH881/88, isolated in 1988 in Thailand), and DEN4 (strain 1228, isolated in 1978 in Indonesia). These chimeric viruses (YF/DEN1, YF/DEN3, and YF/DEN4) replicated to ~7.5 $\log_{10}$ PFU/ml in Vero cells, were not neurovirulent in 3- to 4-week-old ICR mice inoculated by the intracerebral route, and were immunogenic in monkeys. All rhesus monkeys inoculated subcutaneously with one dose of these chimeric viruses (as monovalent or tetravalent formulation) developed viremia with magnitudes similar to that of the YF 17D vaccine strain (YF-VAX) but significantly lower than those of their parent WT viruses. Eight of nine monkeys inoculated with monovalent YF/DEN1 -3, or -4 vaccine and six of six monkeys inoculated with tetravalent YF/DEN1-4 vaccine seroconverted after a single dose. When monkeys were boosted with a tetravalent YF/DEN1-4 dose 6 months later, four of nine monkeys in the monovalent YF/DEN groups developed low levels of viremia, whereas no viremia was detected in any animals previously inoculated with either YF/DEN1-4 vaccine or WT DEN virus. An anamnestic response was observed in all monkeys after the second dose. No statistically significant difference in levels of neutralizing antibodies was observed between YF virus-immune and nonimmune monkeys which received the tetravalent YF/DEN1-4 vaccine or between tetravalent YF/DEN1-4-immune and nonimmune monkeys which received the YF-VAX. However, preimmune monkeys developed either no detectable viremia or a level of viremia lower than that in nonimmune controls. This is the first recombinant tetravalent dengue vaccine successfully evaluated in nonhuman primates.

Dengue is a mosquito-borne flavivirus infection, causing significant morbidity and mortality in tropical areas worldwide (12). There are four dengue virus (DEN) serotypes (1 to 4), all of which cause human illness. Over 2.5 billion people live in areas at risk of the disease worldwide, and 100 million people are affected annually (35, 36, 47). The severe immunopathological form of the disease, dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), is the leading cause of hospitalization of children in Asia. The disease is expanding in distribution and incidence, particularly in the Americas. The United States, infested with *Aedes aegypti*, is vulnerable to introduction of Dengue. The most recent incursion and autochthonous outbreak of dengue occurred in Texas in 1999. Areas of the world recently invaded or at imminent risk include the southern cone (Argentina and Chile), Australia, parts of Africa, southern Europe, and the Middle East.

Mosquito control, as a means of preventing dengue, has been a failure due to expanding urbanization, human population increases, degraded sanitation, competition for financial resources, pesticide resistance, and airline travel, which facilitates movement of viremic travelers. Vaccination has the highest potential as a public health approach that is likely to blunt the increasing incidence and geographic expansion of the disease.

The development of a vaccine against DEN has been a high priority of the World Health Organization for decades (4). An effective vaccine would be used for (i) universal immunization of children in areas of Asia, Latin America, and the Caribbean where dengue is endemic; (ii) protection of foreign travelers and military personnel; and (iii) control of epidemics. Because of the importance of a DEN vaccine for travelers and military personnel in developed countries, DEN vaccines are of interest to the pharmaceutical industry. However, the need for a vaccine extends far beyond such markets, to the people of the most impoverished countries.

Development of a DEN vaccine has been an elusive goal, principally because of the need to simultaneously immunize and induce long-lasting protection against all four DEN serotypes. An incompletely immunized individual, or one in whom antibody titers wanes, may be sensitized to a severe immunopathological disease (DHS/DSS) (15, 26, 45).

The ChimeriVax technology offers a good probability of successful DEN vaccine development. The vaccine attributes include the potential for single-dose application, absent or minimal reactogenicity, extremely durable immunity, reduced potential for interference between the individual components in a tetravalent formulation, and low cost of manufacture. ChimeriVax is a live, attenuated genetically engineered virus,

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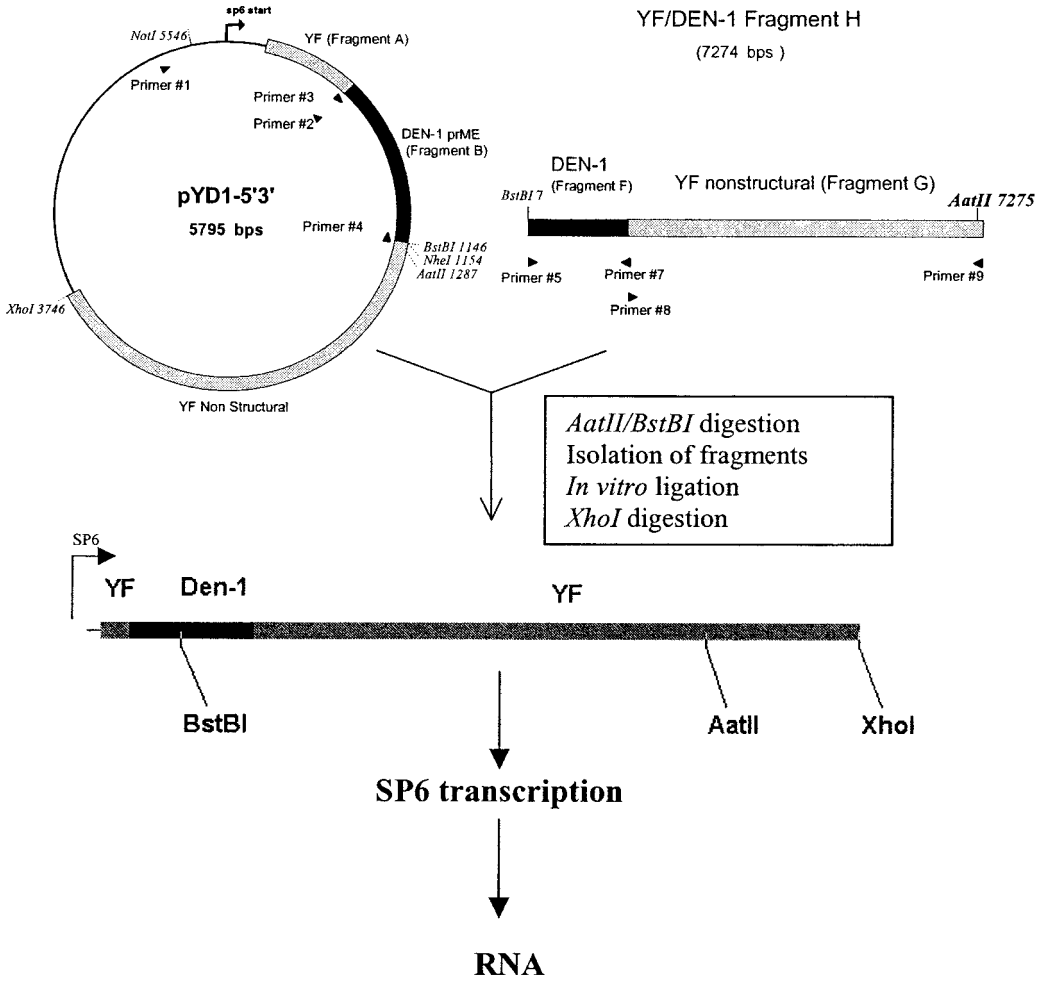

FIG. 1. Synthesis of pYD1-5′3′ and YF/DEN1 fragment H. Overlap-extension PCR was used to fuse the prME sequence of DEN1 strain PUO359 (fragment B) to the YF capsid gene. The resulting PCR product was then subcloned as a *Not*I-*Nhe*I fragment. A silent *Bst*BI site was introduced to facilitate ligation to form the full-length cDNA chimeric clone. Overlap-extension PCR was used to fuse the DEN1 envelope gene to the YF NS genes from pYFM5.2/JE-S (fragment H). In vitro ligation of the *Bst*BI-*Aat*II fragment with plasmid pYD1-5′3′ produced the full-length clone for transcription.

prepared by replacing the genes encoding two structural proteins, the premembrane (prM) and envelope (E) proteins of the yellow fever virus (YF) 17D vaccine strain (YF-VAX) with the corresponding genes of the vaccine target virus, e.g., DEN.

Construction and characterization of YF/DEN2 (in which prME genes of YF 17D were exchanged with those of the PUO218, a Thai strain of DEN serotype 2 [DEN2]) have been described previously (10). In this paper, we describe the construction of chimeric viruses incorporating the prME genes of DEN serotypes 1, 3, and 4. The safety and immunogenicity of these viruses were evaluated in animal models. Studies in rhesus monkeys demonstrated effective simultaneous immunization with all four YF/DEN serotypes.

MATERIALS AND METHODS

Construction of YF-DEN chimeras. (i) Construction of the YF/DEN1 chimera. The two-plasmid system originally constructed to produce a YF infectious clone (43) was the most suitable method for the construction of Japanese encephalitis virus (JE) and DEN2 chimeras (5, 10, 11). However, marked instability of the plasmids encoding the 3′ end of the DEN1 E gene resulted in the use of an overlap-extension PCR alternative. DEN1 prME genes were derived from wild-type (WT) strain PUO359 isolated in 1980 in Thailand and kindly provided by Duane J. Gubler, Centers for Disease Control and Prevention, Fort Collins, Colo., and Robert E. Shope, University of Texas Medical Branch, Galveston. The PUO359 strain was received at passage 1 in C6/36 cells and passaged once more in these cells before the prME and flanking regions were amplified, sequenced, and used for construction of the YF/DEN1 chimera. Sequencing primers were designed based on the DEN1 (strain Philippines 836-1; GenBank accession no. D00503) sequence (6). To create plasmid pYD1-5′3′ (Fig. 1), a reverse transcription (RT)-PCR product encoding DEN1 prM and the 5′ end of E was used as a template along with a fragment encoding the YF 17D C gene derived from plasmid pYF5′3′IV/$JE_{SA14-14-2}$ (5). An overlap-extension PCR resulted in a single fusion product, which was then cloned into an *Nhe*I-*Not*I vector fragment of pYF5′3′IV/$JE_{SA14-14-2}$. To obtain the intermediate part of the chimeric genome, the 3′ end of DEN1 E was fused to the YF nonstructural (NS) genes present in pYFM5.2/$JE_{SA14-14-2}$ by overlap-extension PCR. The resulting amplicon (fragment H) was cloned into plasmid pYD1-5′3′ by in vitro ligation to produce a full-length virus cDNA template for RNA transcription after linearization with *Xho*I. Infectious virus was obtained from Vero cells transfected with the YF/DEN1 mRNA. The titer of virus stock at passage 4 was 7.4 $\log_{10}$ PFU/ml.

(ii) Construction of the YF/DEN3 chimera. The viable YF/DEN3 chimera contained the prME genes of WT DEN3 in place of the corresponding prME region of the genome of YF 17D. The DEN3 parent (strain PaH881/88, Thailand) was originally isolated in 1988 from a patient with classical dengue fever by

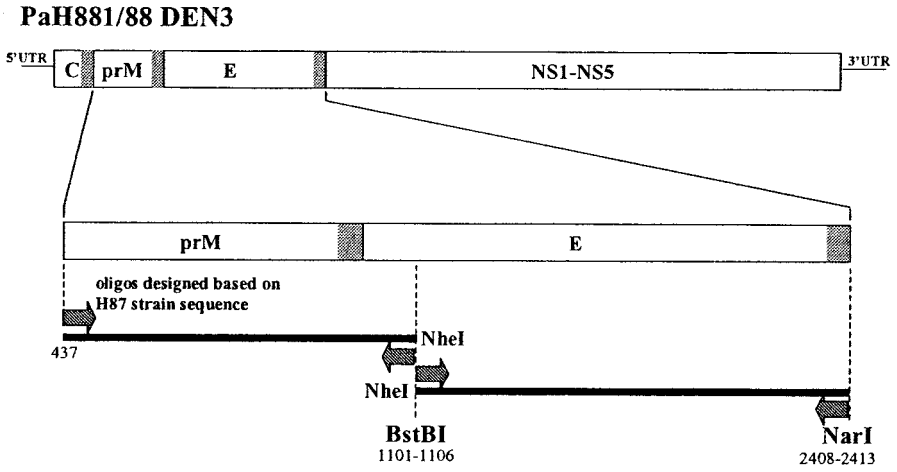

FIG. 2. RT-PCR amplification of the prME region of DEN3 parent strain PaH881/88. The prME region was amplified in two fragments by using H-87 strain-specific primers (arrows). Hydrophobic signals are shadowed. The introduced *Bst*BI site did not result in any amino acid changes, whereas a *Nar*I site introduced a change (Q to G) at the penultimate residue of E. 5′UTR, 5′ untranslated region.

a single amplification in mosquito AP61 cells and kindly provided by Vincent Deubel, Pasteur Institute, Paris, France. This virus was passed once in C6/36 cells before cloning and sequencing. The prME region of the virus was RT-PCR amplified in two adjacent fragments (Fig. 2), using oligonucleotide primers designed based on the Philippine prototype strain of DEN3, H-87, for which the entire nucleotide sequence was known (42). Direct sequencing of these fragments produced a consensus prME sequence of the parent, except for nucleotides 437 to 459, nt 1079 to 1131, and nt 2385 to 2413 (numbering according to the H-87 strain sequence).

The two RT-PCR fragments were then used to replace corresponding JE-specific sequences in plasmids pYFM5′3′IV/$JE_{SA14-14-2}$ and pYFM5.2/$JE_{SA14-14-2}$ (5), which resulted in plasmids 5′3′/Den3 and 5.2/Den3 (Fig. 3). An extra *Xho*I site in 5′3′/Den3 (in the E gene of DEN3) was ablated by silent mutagenesis, resulting in plasmid 5′3′/Den3/ΔXho. All plasmids were sequenced to ensure the absence of any additional mutations. Similar to the situation with YF/DEN1, technical difficulties were encountered during cloning of plasmid 5.2/Den3 due to its toxicity in *Escherichia coli*. The selected clone of the plasmid (clone 26) had no mutations except for a single nucleotide deletion at the 3′ end of the DEN3 insert. Therefore, to correct the deletion, a three-fragment ligation was used (Fig. 3). The DEN3 part of 5.2/Den3 was amplified by PCR using the clone 26 template with high-fidelity ExTaq polymerase (TaKaRa) (the opposite primer corrected the deletion) and digested with *Bst*BI and *Nar*I. The *Nar*I-*Aat*II fragment was derived from pYFM5.2/$JE_{SA14-14-2}$. The two fragments were ligated with the large *Bst*BI-*Aat*II fragment of 5′3′/Den3/ΔXho. Ligation products were digested with *Xho*I and transcribed in vitro with SP6 RNA polymerase. Subsequently, the DEN3-specific *Bst*BI-*Nar*I fragment was individually cloned without any mutations resulting, in plasmid pCL/Den3E. Thus, YF/DEN3 has a complete plasmid backup and can be reproduced in the future without a PCR step. The chimera was obtained by transfection of Vero cells with the RNA transcripts and then amplified in Vero cells. The chimera peak titers at both passage 2 (P2) and P4 were 6.3 $\log_{10}$ PFU/ml.

(iii) Construction of the YF/DEN4 chimera. To construct the YF/DEN4 chimera, we used the standard two-plasmid system (5, 10, 43). The source of DEN4 prME genes was strain 1228, isolated in 1978 from a classical dengue fever case in Indonesia, passaged twice in mosquitoes by intrathoracic inoculation and once in C6/36 cell culture (the virus was a gift from Duane J. Gubler and Robert E. Shope). The virus was passaged once in C6/36 cells; viral RNA was extracted from infected cells; the prME region was amplified, sequenced, and used to construct a YF/DEN4 chimera. The DEN4 prME region was first amplified and sequenced using primers mostly derived from DEN4 (Caribbean strain 814669; GenBank accession no. M14931) (32). The sequence data created were used to design primers for synthesis of cDNA and assembly of the two-plasmid system of DEN4 (i.e., by replacing the corresponding prME sequences of $JE_{SA14-14-2}$ with those of DEN4 in each plasmid). First, a PCR product encoding DEN4 prM and the 5′ end of E (Fig. 4, fragment B) was used along with a template encoding the C gene of YF 17D (fragment A) derived from plasmid pYF5′3′IV/$JE_{SA14-14-2}$ in an overlap-extension PCR. This resulted in a single fusion product, which was then cloned into an *Nhe*I-*Not*I fragment of pYFM5′3′IV, where $JE_{SA14-14-2}$ sequences were deleted to create plasmid pYD4-5′3′. The 3′ end of the DEN4 E protein gene was also amplified (fragment C) and then cloned into plasmid pYFM5.2/

$JE_{SA14-14-2}$ as an *Nhe*I-*Sfo*I fragment replacing $JE_{SA14-14-2}$ sequences with that of DEN4 to create plasmid pYD4-5.2. In vitro ligation of the two plasmids resulted in a full-length virus cDNA template of YF/DEN4 for RNA transcription. The titer of virus stock at P3 posttransfection was 7.1 $\log_{10}$ PFU/ml. The plaque analysis of this virus revealed a mixed population of small to large plaques in Vero cells. One large and one small plaque were subjected to three rounds of plaque purifications (with one amplification between each round). Plaque-puri-

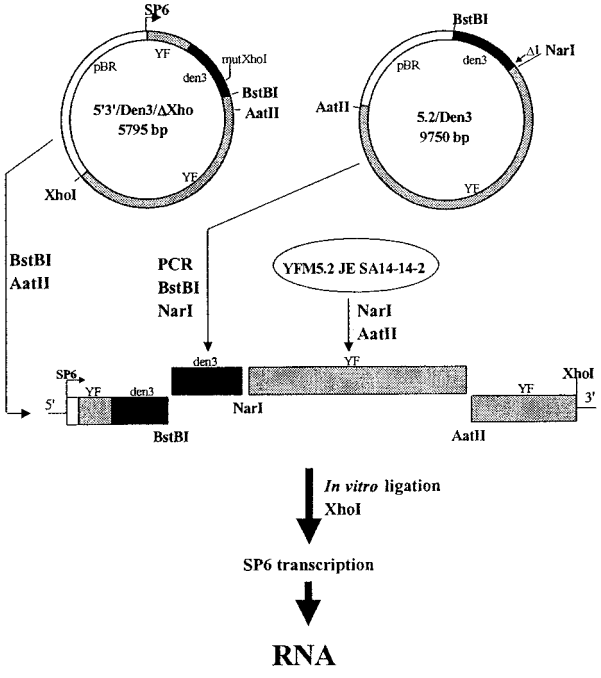

FIG. 3. Construction of the YF/DEN3 chimera. YF- and DEN3-specific sequences are shown as shadowed and black boxes, respectively. The chimeric YF/DEN3 genome was reconstituted by in vitro ligation of three DNA fragments (see Materials and Methods). mut*Xho*I, mutated *Xho*I site.

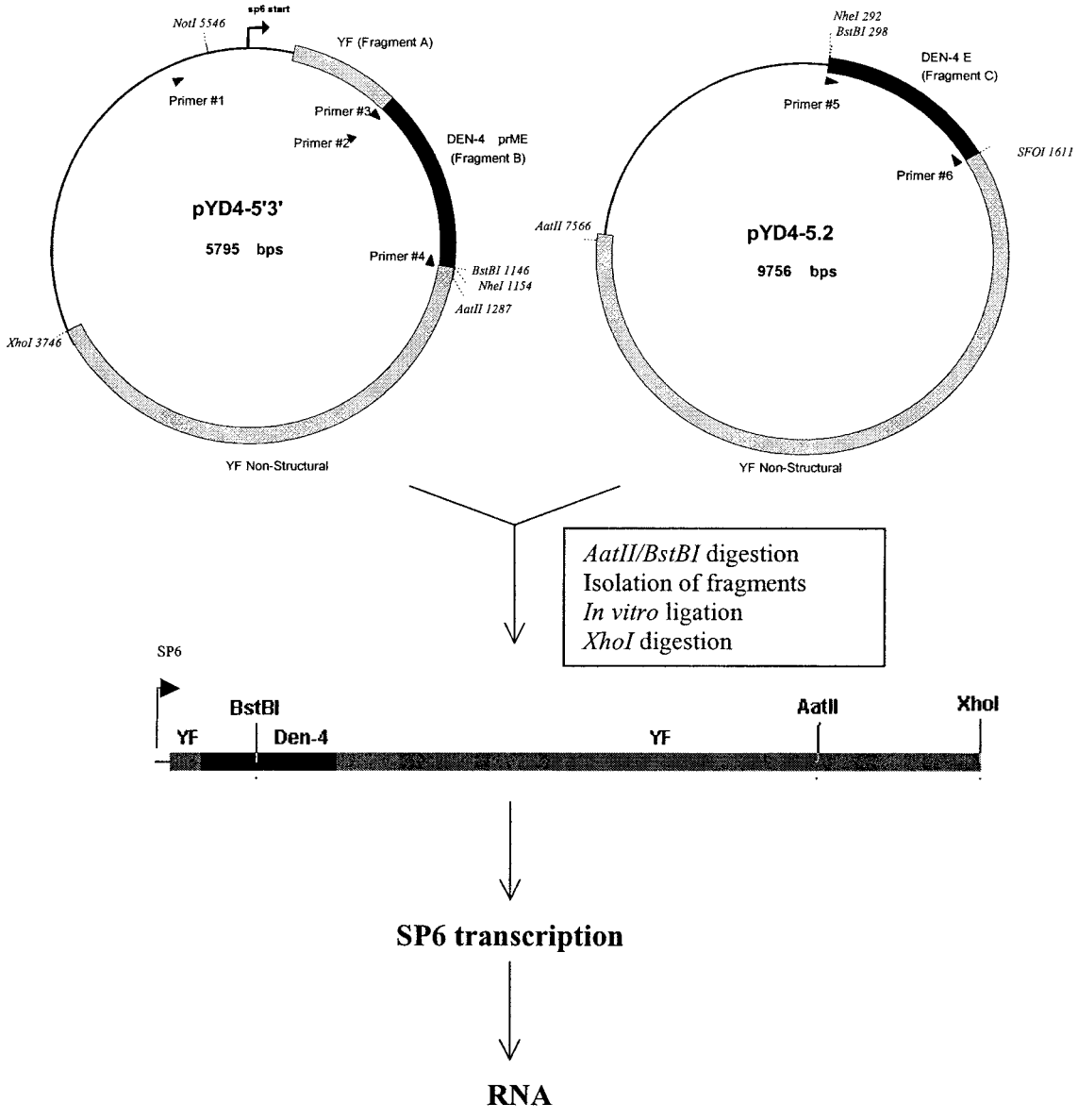

FIG. 4. YF/DEN4 two-plasmid system. Overlap-extension PCR was used to fuse the prME sequence of DEN4 strain 1228 (fragment B) to the YF capsid gene. The resulting PCR product was then subcloned as a *Not*I-*Nhe*I fragment into plasmid pYF5′3′IV/JE-S. A silent *Bst*BI site was introduced to facilitate ligation to form the full-length cDNA chimeric clone. For synthesis of pYD4-5.2, an overlap-extension PCR was used to fuse the DEN4 envelope gene to the YF NS genes in plasmid pYFM5.2/JE-S. An *Sfo*I site replaced the *Nar*I site during subcloning of the fragment into plasmid pYD4-5.2. In vitro ligation of the *Bst*BI-*Aat*II fragment with plasmid pYD4-5′3′ produced a full-length clone for transcription.

fied viruses were sequenced at P8 (prME regions), and their growth kinetics in Vero cell were compared to the kinetics of their uncloned chimeric parent virus (at P3 posttransfection) (see below). The purified large-plaque viruses (at P8) were used in all monkey experiments.

Cells and viruses. Vero cells were provided by Aventis Pasteur (Lyon, France) and were used between P141 and P151 for transfection with chimeric in vitro RNA transcripts and between P143 and P170 for other purposes such as plaque assays and neutralization tests. C6/36 cells were obtained from the American Type Culture Collection, Manassas, Va. In addition to the four YF/DEN chimeras described above, YF-VAX (Aventis Pasteur) and three WT viruses, DEN1 (strain PUO359, Thailand), DEN3 (strain PaH881/88, Thailand), and DEN4 (strain 1228, Indonesia), were used in this study.

Animal studies. All studies were carried out under an Institutional Animal Care and Use Committee-approved protocol in accordance with the USDA Animal Welfare Act (9 CFR parts 1 to 3) as described in the *Guide for Care and Use of Laboratory Animals* (41).

For studies of neurovirulence in mice, groups of five 4-week-old outbred ICR mice (Taconic Farm, Inc. Germantown, N.Y.) were inoculated by the intracerebral (i.c.) route with ~5 $\log_{10}$ PFU of YF/DEN chimera, parent WT DEN (from which prME genes were derived for chimera construction), or YF-VAX. Animals were observed for 21 days, and any animals found in an advanced moribund stage were euthanized.

Experiments with monkeys were performed at the Tulane Regional Primate Research Center (Covington, La.) in healthy, young adult, colony-reared Indian rhesus monkeys (*Macacca mulatta*). The goals of these experiments were to determine (i) viremia and immunogenicity of YF/DEN chimeras compared to those of their parent viruses (WT DEN and YF 17D) after primary immunization, (ii) whether a second dose of the tetravalent vaccine would increase the

level of neutralizing antibodies to individual viruses, and (iii) whether there is any interference between YF-VAX and the YF/DEN1-4 tetravalent vaccine.

(i) Viremia and immunogenicity profiles of ChimeriVax-DEN (experiment a). Twenty-seven male monkeys weighing between 5.8 and 8.35 kg were divided randomly into eight groups and immunized subcutaneously (s.c.) into the right arm over the area of the deltoid muscle with 0.5 ml of test vaccine. Groups 1 to 3 and 5 to 7 (three monkeys per group) were immunized with 4.7 $\log_{10}$ PFU of YF/DEN1, YF/DEN3, YF/DEN4, WT DEN1 (strain PUO359), WT DEN3 (strain PaH881/88), and WT DEN4 (strain 1228), respectively. Monkeys in group 4 ($n = 6$) received a mixture of equal concentrations (4.7 logs/0.5 ml) of each of the four YF/DEN chimeras (total, 5.3 $\log_{10}$PFU/2 ml) administered into the right and left arms (1 ml into each arm). The eighth group of monkeys ($n = 3$) received 0.5 ml of undiluted YF-VAX (5.0 $\log_{10}$ PFU). The remaining inocula were frozen for back titration. Blood from the femoral vein was collected from all animals under anesthesia prior to immunization, then daily for the following 10 days for determination of viremia, and on days 15, 30, and 79 for assessment of neutralizing antibody titers.

(ii) Booster immunization with tetravalent chimeric DEN vaccine (experiment b). Six months after primary immunization, six additional naive monkeys (weighing between 2.6 and 3.9 kg) were added to the experiment as an unimmunized control group (group 9). All animals ($n = 27$) that had been immunized as described above plus the six unimmunized control monkeys received 2.0 ml of YF/DEN1-4 vaccine (a tetravalent mixture containing 5.0 $\log_{10}$ PFU each of YF/DEN1, YF/DEN2, YF/DEN3, and YF/DEN4) by the s.c. route into both arms (1 ml per arm). Inocula were frozen for back titration. Blood was collected immediately prior to inoculation, then daily for the next 12 days for determination of viremia, and on day 30 for assessment of neutralizing antibody titers. Animals were released from the study on day 31.

(iii) Preimmunity to YF/DEN1-4 tetravalent vaccine. Ten monkeys (four monkeys from group 4 and six monkeys from group 9, which had previously received two and one doses of tetravalent vaccine, respectively, in experiments a and b) were recaptured 6 months after their release. These animals, together with a group of four naive monkeys (as unimmunized controls [group 1]), were inoculated s.c. with 0.5 ml of undiluted YF-VAX (~5.0 $\log_{10}$ PFU). Inocula were frozen for back titration. Blood was drawn immediately prior to immunization, then daily for 10 days for determination of viremia, and on day 30 for determination of neutralizing antibody titers.

Throughout the study, animals were observed daily for clinical signs. All animals remained healthy and appeared normal with respect to eating and behavioral activity. However, 1 week after the second dose of vaccine (>6 months after the original immunization), a minimal rash was observed in three monkeys (monkey T791 from group 2, YF/DEN1; monkey T264 from group 7, WT DEN4; and monkey T354 from group 8, tetravalent vaccine) confined to the upper arms (monkeys T791 and T264) and the left shoulder (monkey T354). Four days later, the rash on all monkeys had resolved, and no other clinical signs were recorded until the monkeys were released 7 months post-primary immunization.

Viremia and neutralization assays. Viremia and plaque reduction neutralization tests were determined on Vero cells, using agarose double overlay and neutral red as described previously (10, 11, 38). Virus titers in serum were determined by direct plaquing in Vero cells using undiluted, 2- and 10-fold dilutions of sera. The level of virus detection was 0.7 $\log_{10}$ PFU/ml. Neutralizing antibody titers were determined on heat-inactivated (56°C, 30 min) sera without the addition of complement (38).

Serotype identification by RT-PCR amplification and immunocytochemical focus-forming assay. (i) Serotype identification by RT-PCR–restriction enzyme assay. Individual plaques from sera of monkeys immunized with tetravalent YF/DEN1-4 vaccine were amplified once in Vero cells. Virions RNAs were extracted (from 125 μl of supernatants of infected cells) using TRI Reagent-LS (Molecular Research Center, Inc.) according to the manufacturer's procedure. To amplify the prME regions, each extracted RNA was used as a template in a 25-μl single-tube RT-PCR (Titan; Roche), according to the manufacturer's protocol, using YF0.2+ (5′-ATGGTACGACGAGGAGTTCGC) and KP5.2/1.66− (5′-CTCTAAATATGAAGATACCATC) YF-specific primers flanking the DEN-specific prME genes of the chimeras. Following amplification, a 2-μl aliquot of each RT-PCR mixture containing approximately 0.5 μg of a 2.37-kb fragment was digested with *Hin*dIII in a 15-μl volume. Digestion products were resolved in a 1% agarose gel in the presence of ethidium bromide, and the DEN type specificity of each fragment was visually identified. DEN1-, DEN2-, and DEN3-specific fragments were digested only once, producing two bands of 1972 and 402 bp for DEN1, 1,429 and 945 bp for DEN2, and 1,217 and 1,151 bp for DEN3 chimeras. The DEN4-specific fragment was not digested with *Hin*dIII.

(ii) Serotype identification by immunocytochemical focus-forming assay. Individual plaques from sera of monkeys immunized with tetravalent YF/DEN1-4 vaccine were amplified once in Vero cells; supernatants were harvested and inoculated into 4 wells of a 12-well plate seeded with Vero cells. After 1 h of virus adsorption at 37°C, wells were overlaid with minimal essential medium supplemented with 10% fetal bovine saline, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.75% methylcellulose (Sigma) and incubated for 3 days at 37°C. Cell monolayers were fixed for 1 h by addition of 1 ml of 4% formalin, wells were washed with tap water, and 1 ml of blocking/permeabilizing buffer (2.5% nonfat dry milk, 0.05% Tween 20, and 0.5% Triton X-100 in phosphate-buffered saline) was added to each well. After 15 min at room temperature, blocking/permeabilizing buffer was removed and 0.5 ml of virus-specific primary antibody was added to wells. Primary monoclonal antibodies (MAbs) used were D2-1F1-3 and D6-8A1-12 (provided by John Roehrig, Centers for Disease Control and Prevention, Fort Collins, Colo.), specific for DEN1 and DEN3, respectively, and 3H5-1 and 1H10 (American Type Culture Collection), specific for DEN2 and DEN4, respectively. MAbs were produced by growth of hybridoma cells in tissue culture and diluted in blocking/permeabilizing buffer. Supernatant fluids of tissue culture flasks (D2-1F1-3 and D6-8A1-12) were diluted 1:10, and ammonium sulfate-precipitated material from CL-1000 high-density culture flasks (Integra Biosciences, Ijamsville, Md.) was diluted 1:4,000 (3H5-1) or 1:1,000 (1H10). Following 1 h incubation at room temperature on a rotating platform, plates were washed three times with phosphate-buffered saline–0.05% Tween 20 (wash buffer), and 0.5 ml of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Southern Biotechnology Associates, Birmingham, Ala.) diluted 1:500 in blocking/permeabilizing buffer was added to each well. Following 1-h incubation at room temperature, plates were washed three times with wash buffer, and antibody-bound foci of infection were developed (by addition of 5-bromo-4-chloro-3-indolylphosphate–nitroblue tetrazolium [Sigma Chemical Co., St. Louis. Mo.] containing 4 mM levamisole [Sigma] to block endogenous alkaline phosphatase activity) and counted.

Sequencing. WT DEN strains were grown in C6/36 cells, while chimeric viruses were grown in Vero cells. Supernatants were harvested 7 days (from C6/36 cells) or 3 to 4 days (from Vero cells) postinfection, and viruses were sequenced across prME regions by automated sequencing, essentially as described previously (10), using a collection of DEN-specific primers.

Statistical analysis. Differences in responses among groups and between two groups were analyzed for significance using one-way analysis of variance (ANOVA) and *t* tests, respectively (JMP software version 4.0.2).

RESULTS

Sequence of YF/DEN chimeras. In addition to the amino acid change from Q to G at E protein 494 position (E494; E492 for YF/DEN3), which were intentionally created for insertion of a *Nar*I restriction site at the E/NS1 junction, other mutations were observed in the chimeric viruses (Table 1). DEN1 chimeras differed from the parental PUO359 strain at M39 (H to R) and E204 (K to R). An amino acid difference at the membrane-spanning domain (E484, I to V) separated DEN2 chimeras and their parent PUO218 strain (Table 1) (10). There was one amino acid difference between the DEN3 chimera and the parental strain, PaH881/88 (E489 A to V), also in the membrane-spanning region (primer region) of the E proteins. Sequencing of the DEN4 chimeras revealed mutations at M43 (A to T), E4 (V to I), E56 (L to F), and E437 (H to Y) compared to the parental DEN4 1228 strain. Three additional amino acid substitutions in the E proteins of the large-plaque phenotype of YF/DEN4 chimeras distinguished them from the small-plaque phenotype: E277 (H to N), E366 (N to S), and E437 (H to Y) (Table 1).

Growth of chimeric viruses in Vero cells. Vero cells were infected with chimeric viruses at a multiplicity of infection of ~0.001. Aliquots of cell culture medium were taken on days 1 to 5 and tested in a Vero cell plaque assay as described previously (38). The peak titers were 8.0, 7.9, 6.3, and 7.5 $\log_{10}$ PFU/ml for YF/DEN1, YF/DEN2, YF/DEN3, and YF/DEN4

TABLE 1. Nucleotide and amino acid differences (in the prME region) between chimeric and parent WT viruses

Virus, cell passage	Gene	Nucleotide no.[a]	Nucleotide change	Amino acid change	Amino acid no.[b]	Comment
YF/DEN1, Vero P4	M	389	A to G	H to R	39	Present in plasmid
	E	1109	A to G	K to R	204	
	E	1978–1980	CAG to GGC	Q to G	494	Site used to insert *Nar*I
YF/DEN2, Vero P3	E	1948–1950	ATT to GTC	I to V	484	Likely was present in plasmid (9), GenBank accession no. AF038402)
	E	1978–1980	CAG to GGG	Q to G	494	Site used to insert *Nar*I
YF/DEN3, Vero P4	E	1964	C to T	A to V	489	
	E	1972–1974	CAA to GGC	Q to G	492	Site used to insert *Nar*I
YF/DEN4, Vero P3 (uncloned)	M	400	G to A	A to T	43	
	E	508	G to A	V to I	4	
	E	666	A to T	L to F	56	
	E	1807	C to T	H to Y	437	
	E	1978–1980	CAA to GGC	Q to G	494	Site used to insert *Nar*I
YF/DEN4, Vero P8 (large plaque)	M	400	G to A	A to T	43	
	E	666	A to T	L to F	56	
	E	1327	C to A	H to N	277	
	E	1595	A to G	N to S	366	
	E	1807	C to T	H to Y	437	
	E	1978–1980	CAA to GGC	Q to G	494	Site used to insert *Nar*I
YF/DEN4, Vero P8 (small plaque)	M	400	G to A	A to T	43	
	E	666	A to T	L to F	56	
	E	1978–1980	CAA to GGC	Q to G	494	Site used to insert *Nar*I

[a] From beginning of the prME gene.
[b] From N terminus of the protein (M or E).

(uncloned), respectively. The peak titer of large-plaque YF/DEN4 (7.7 $\log_{10}$ PFU/ml, on day 4) was similar to that of its uncloned parent virus (7.5 $\log_{10}$ PFU/ml, on day 4). The small-plaque virus grew slower and reached its peak titer 1 day later (7.2 $\log_{10}$ PFU/ml on day 5) (data not shown).

Neurovirulence of chimeric viruses for mice. Four-week-old outbred ICR mice were inoculated with either chimeric (YF/DEN1, YF/DEN3, and YF/DEN4) or parent WT (DEN1, strain PUO359; DEN3, strain PaH881/88; and DEN 4, strain 1228) virus by the i.c. route. Inoculated doses (determined by back titration of retained samples) were 5 $\log_{10}$ PFU/0.03 ml for all viruses except YF/DEN3, which was used at a dose of 4 $\log_{10}$ PFU/0.03 ml. No mice died or showed any sign of illness during the 21-day observation period, demonstrating that the YF backbone in the chimeras did not increase the neurovirulence of these viruses (the YF 17D vaccine strain is lethal for mice of all ages inoculated by the i.c. route [11]).

Viremia and immunogenicity of chimeric viruses in rhesus monkeys. (i) Primary immunization with YF/DEN chimeras. All monkeys became viremic after inoculation with chimeric viruses (as monovalent or tetravalent vaccines), WT DEN, or YF-VAX. The peak virus titers (ranging from 0.7 to 1.4 $\log_{10}$ PFU/ml) and duration of viremia (1 to 3 days) for monovalent chimeric viruses were generally lower than those of monovalent WT parent viruses (mean peak titers of 2.2 to 3.0 $\log_{10}$ PFU/ml and mean duration of 2.7 to 3.3 days) (Table 2). The peak virus titers and duration of viremia for the tetravalent vaccine or YF-VAX were similar to those for chimeric monovalent vaccines. To determine which chimeric viruses are present in viremic monkeys, sera of monkeys in group 4 (tetravalent group) were tested by RT-PCR and *Hin*dIII restriction analysis, as well as by immunocytochemical focus-forming assay using MAbs specific for individual serotypes. Both techniques produced similar results, identifying the presence of three of the four chimeras (YF/DEN2, YF/DEN3, and YF/DEN4) on different days postinoculation. The most frequently isolated virus was YF/DEN2, whereas YF/DEN1 could not be isolated on any days using both techniques (Table 2).

The level of neutralizing antibody titers in monkeys immunized with monovalent chimeric viruses was lower than that for YF-VAX or WT DEN. Monkey T747 in the chimeric DEN3 group did not seroconvert (the 50% plaque reduction neutralizing titer [PRNT] was <1:20, the lowest serum dilution tested), and monkey T230 in chimeric DEN4 group had a borderline titer of 1:10 (Table 3). All six monkeys in the tetravalent group seroconverted to all four DEN viruses, except monkey T805, which did not seroconvert to DEN4 virus (Table 4). The strongest response in all six monkeys was directed to YF/DEN2 viruses (geometric mean titer [GMT], >1,015).

(ii) Viremia and immunogenicity of YF/DEN chimeras after secondary immunization. All monkeys described in experiment a (Materials and Methods), together with 6 naive animals, were given a dose of YF/DEN1-4 tetravalent vaccine (~5.0 $\log_{10}$ PFU of each serotype) 6 months after primary immunization. None of the monkeys immunized previously with monovalent WT DEN or tetravalent YF/DEN1-4 vaccine became viremic, whereas all six naive monkeys became viremic for a mean duration of 4.7 days and mean peak titer of 1.2 $\log_{10}$ PFU/ml (Table 5). One of three animals previously immunized with monovalent YF/DEN3 or YF/DEN4 and two of three animals previously immunized with YF/DEN1 or YF-VAX became viremic after the booster dose. Generally, viremia levels in preimmune monkeys were lower than in the naive control group, indicating a degree of cross-protection between the

TABLE 2. Viremia[a] in rhesus monkeys after primary immunization with monovalent or tetravalent YF/DEN vaccine, WT parent DEN virus, or YF-VAX

Group	Monkey	Virus [strain] (dose, $\log_{10}$ PFU)[b]	No. viremic/ no. tested (%)	Mean		Serotype detected[e] (day[s])
				Peak titer[c] (SD)	Duration[d] (SD)	
1	T340 T791 T744	YF/DEN1 (4.3)	3/3 (100)	0.7 (0)	1.3 (0.6)	NT[f]
2	T747 T383 T144	YF/DEN3 (3.6)	3/3 (100)	1.3 (0.4)	1 (0)	NT
3	T230 T260 T548	YF/DEN4 (3.8)	3/3 (100)	1.4 (1)	3 (2)	NT
4	T401 V744 BA28 T805 T354 T036	YF/DEN1–4 (5.3 total)[g]	6/6 (100)	1.5 (0.5)	3.3 (2.2)	2 (2, 3, 5, 6, 10), 4 (2) 2 (9) 4 (9–10) 2 (1–2, 4–5, 9), 3, 4 (2) 2 (7) 2 (2, 4, 6, 8–9), 3 (4, 7–8)
5	T383 T144 T547	WT DEN1 [PUO359] (3.9)	3/3 (100)	3.0 (0.9)	3.3 (2.1)	NT
6	T502 T600 T602	WT DEN3 [PaH881/88] (5.2)	3/3 (100)	2.8 (0.5)	2.7 (1.1)	NT
7	T264 T529 T427	WT DEN4 [1228] (3.8)	3/3 (100)	2.2 (0.15)	3.3 (0.6)	NT
8	BA29 V576 V202	YF-VAX (5.0)	3/3 (100)	1.9 (0.6)	2.3 (1.1)	NT
P[h]				0.03	0.18	

[a] Viremia for groups immunized with YF/DEN chimeras was measured in a standard plaque assay using Vero cells and an agarose double overlay containing neutral red, whereas viremia for groups immunized with WT DEN was measured using C6/36 cells and an immunocytochemical focus assay (10).
[b] Determined by back titration of inocula.
[c] $\log_{10}$ PFU per milliliter.
[d] Days.
[e] Determined by RT-PCR and *Hin*dIII maps as well as immunocytochemical focus-forming assay using MAbs (see Materials and Methods).
[f] NT, not tested.
[g] The concentration of each serotype in the tetravalent mixture was determined by back titration of inocula using an immunocytochemical focus-forming assay. The doses of YF/DEN1, YF/DEN2, YF/DEN3, and YF/DEN4 were 4.4, 4.2, 4.0, and 3.7 $\log_{10}$ PFU, respectively.
[h] Differences in peak and duration of viremia across groups were measured using one-way ANOYA test (JMP software, version 4.0.2).

different vaccine viruses. Viremia lasted from 1 to 5 days, and peak titers varied from 1 to 2.4 $\log_{10}$ PFU/ml (Table 5).

Neutralizing antibody titers were determined against all four YF/DEN chimeras in sera collected immediately before (day 180) and 30 days after the booster immunization. As expected, neutralizing antibodies in monkeys given a single serotype of chimeric or WT virus were relatively specific prior to boosting. Neutralizing antibody titers were higher in monkeys previously immunized with monovalent WT DEN than monovalent chimeras. For example, the GMTs in monkeys previously immunized with WT DEN1 virus were 6,451 against homologous virus (WT DEN1) and 254, 403, and 101 against heterologous viruses (WT DEN2, WT DEN3, and WT DEN4 viruses, respectively). In contrast, the GMTs for monkeys in the YF/DEN1 group were 160 against homologous and 3, 2, and 1 against heterologous viruses (YF/DEN2, YF/DEN3, and YF/DEN4 viruses, respectively) (Table 6). In addition, all monkeys immunized previously with one YF/DEN serotype showed anamnestic responses to the same serotype and also seroconverted to the other three serotypes (contained in the tetravalent booster dose). Also, monkey T747 in the YF/DEN3 group and monkey T230 in YF/DEN4 group, which had not seroconverted or had developed a low level of neutralizing antibodies after the primary immunization, developed high titers (5,120 and 160) against YF/DEN3 and YF/DEN4, respectively (Table 6). Generally, all six monkeys in the tetravalent group were boosted against all four serotypes, with some exceptions; in monkey V744, the 50% PRNTs against all four serotypes were not significantly changed after the booster dose, and neutralizing antibody titers against YF/DEN3 in monkey BA28 remained unchanged (1:160) after the booster dose. Despite the undetectable viremia in monkeys previously immunized with WT DEN, these monkeys produced high level of neutralizing antibodies against all four YF/DEN chimeras.

Vector immunity. Since chimeric DEN vaccine candidates were constructed on the backbone of YF 17D, it was important to determine whether YF immunity would restrict immunogenicity of chimeric viruses or vice versa. Two experiments were carried out; in the first experiment, monkeys with or without YF immunity were inoculated with the YF/DEN1-4 tetravalent vaccine; in the second experiment, monkeys without prior immunization or with prior YF/DEN immunization (one or two

TABLE 3. Neutralizing antibody titers (50%) in rhesus monkeys immunized with YF/DEN monovalent vaccine, WT DEN, or YF-VAX

Group	Monkey	Virus [strain] (dose, $\log_{10}$ PFU)[b]	50% PRNT by postimmunization day[a]:	
			30	79
1	T340	YF/DEN1 (4.3)	80	40
	T791		640	320
	T744		2,560	1,280
GMT (SD)			508 (1,300)	254 (650)
2	T747	YF/DEN3 (3.6)	<20	<20
	T383		80	160
	T144		640	160
GMT (SD)			37 (348)	29 (92)
3	T230	YF/DEN4 (3.8)	10	<10
	T260		2,560	5,120
	T548		2,560	320
GMT (SD)			403 (1,472)	117 (2,868)
5	T725	WT DEN1 [PUO 359] (3.9)	2,560	5,120
	T613		2,560	5,120
	T547		2,560	10,240
GMT (SD)			2,560 (0)	6,450 (2,956)
6	T502	WT DEN3 [PaH881/88] (5.2)	640	320
	T600		1,280	2,560
	T602		1,280	1,280
GMT (SD)			1,016 (369)	1,016 (1,124)
7	T264	WT DEN4 [1228] (3.8)	1,280	2,560
	T529		1,280	2,560
	T427		10,240	2,560
GMT (SD)			2,560 (5,173)	2,560 (0)
8	BA29	YF-VAX[b](5.0)	2,560	1,280
	V576		5,120	2,560
	V202		2,560	1,280
GMT (SD)			3,225 (1,478)	1,613 (739)

[a] Titers of sera on day 0 were <10 in all animals.
[b] Determined by back titration of inocula.

doses of vaccine) were inoculated with YF-VAX. The interval between the two vaccines (YF/DEN1-4 and YF-VAX) was 6 months.

Immunogenicity of YF/DEN1-4 tetravalent vaccine in YF-immune monkeys. Two of three YF-immune and all six nonimmune monkeys developed viremia after immunization with YF/DEN1-4 tetravalent vaccine. The mean virus peak titers and duration of viremia in YF-immune monkeys (1.3 $\log_{10}$ PFU/ml and 1.5 days, respectively) were lower than in nonimmune monkeys (2.4 $\log_{10}$ PFU/ml and 4.7 days, respectively) (Table 7). Chimeric YF/DEN1 and YF/DEN2 were isolated from YF-immune monkeys up to 3 days after inoculation, whereas YF/DEN2, YF/DEN3, and YF/DEN4 could be isolated from nonimmune monkeys up to 11 days after inoculation. The peak virus titers and durations of viremia were significantly higher in nonimmune than in YF-immune monkeys *P* values for both peak titers and duration of viremia were 0.007 as determined by *t* test (Table 7).

All monkeys developed neutralizing antibodies to all four DEN serotypes. There was no statistical difference in titers

TABLE 4. Neutralizing antibody titers (50%) in rhesus monkeys immunized with YF/DEN1–4 tetravalent vaccine (total dose, 5.3 $\log_{10}$ PFU)

Group	Monkey	Neutralizing titer (against YF/DEN) by post immunization day:							
		YF/DEN1		YF/DEN2		YF/DEN3		YF/DEN4	
		30	79	30	79	30	79	30	79
4	T401	640	160	>5,120	5,120	40	40	80	80
	V744	80	320	1,280	1,280	80	160	20	320
	BA28	160	80	640	2,560	80	80	80	320
	T805	320	40	320	160	40	<20	10	10
	T354	80	80	640	320	80	40	40	40
	T036	80	80	1,280	1,280	320	80	20	80
GMT (SD)		160 (223)	101 (102)	>1,015 (1,792)	1,015 (1,844)	80 (106)	34 (54)	32 (31)	80 (141)

TABLE 5. Viremia[a] in rhesus monkeys after secondary immunization with YF/DEN1–4 tetravalent vaccine (total dose, 5.4 $\log_{10}$ PFU)

Group	Monkey	Primary immunization [strain] (dose, $\log_{10}$ PFU)[b]	No. viremic/ no. tested (%)	Mean		Serotype detected[e] (day[s])
				Peak titer[c] (SD)	Duration[d] (SD)	
1	T340	YF/DEN1 (4.3)	2/3 (67)	1.5 (0.2)	1.0 (0)	2 (2)
	T791					2 (2)
	T744					
2	T747	YF/DEN3 (3.6)	1/3 (33)	2.3 (0)	5 (0)	1 (1, 2), 2 (1, 3, 7, 9, 11)
	T383					
	T144					
3	T230	YF/DEN4 (3.8)	1/3 (33)	1.0 (0)	3 (0)	2 (2, 3, 4)
	T260					
	T548					
4	T401	YF/DEN1–4 (5.3 total)	0/6 (0)			
	V744					
	BA28					
	T805					
	T354					
	T036					
5	T725	WT DEN1 [PUO359] (3.9)	0/3 (0)			
	T613					
	T547					
6	T502	WT DEN3 [PaH881/88] (5.2)	0/3 (0)			
	T600					
	T602					
7	T264	WT DEN4 [1228] (3.8)	0/3 (0)			
	T529					
	T427					

[a–d] See Table 2, footnotes *a* to *d*.
[e] Determined by RT-PCR followed by *Hin*dIII digestion.

against all serotypes between YF-immune and nonimmune monkeys except for YF/DEN3. The titer of neutralizing antibodies against YF/DEN3 was significantly higher in YF-immune than nonimmune monkeys (GMT of 350 versus 36, $P = 0.013$, t test) (Table 8).

Immunogenicity of YF-VAX in monkeys previously given YF/DEN1-4. Monkeys with or without background immunity to YF/DEN1-4 tetravalent vaccine (unimmunized or immunized with one dose [Table 8] or two doses [Table 4] of vaccine) were inoculated with a standard human dose (5 $\log_{10}$ PFU) of YF-VAX. Viremia (measured from days 1 to 12 postinoculation) and neutralizing titers (measured 30 days postimmunization) are shown in Table 9. No viremia was detected in any of four monkeys previously inoculated with two doses of YF/DEN1-4 tetravalent vaccine, whereas minimal viremia (1 $\log_{10}$ PFU/ml), on a single day, was observed in two of six monkeys, which previously had received only one dose of YF/DEN1-4 tetravalent vaccine. In contrast, three of four monkeys in the control group became viremic (peak titers ranged from 2.1 to 2.3 $\log_{10}$ PFU/ml, and mean duration was 3 to 4 days) (Table 9). Mean peak and duration of viremia in unimmunized monkeys were significantly higher ($P = 0.01$ and 0.006, respectively) than immunized animals. Despite lack of viremia in some preimmune monkeys, all developed high titers of neutralizing antibodies against YF 17D. Levels of neutralizing antibody titers appeared to be similar across groups ($P = 0.16$) (Table 9).

DISCUSSION

YF 17D has been used successfully as a vector for construction of YF/JE (5) and YF/DEN2 (3, 10, 55) chimeras. We previously described safety and immunogenicity of YF/JE and YF/DEN2 chimeras in mice and monkeys (10, 11, 38, 39), and we recently completed a phase 1 clinical trial of a YF/JE chimera in human volunteers (T. P. Monath et al., unpublished data). In this paper, we describe constructions and evaluations of three additional chimeras as potential vaccine candidates against DEN serotypes 1, 3, and 4.

The genetic construction of the chimeric viral genome and replacement of DEN genes was accomplished based on the full-length clone of YF 17D/JE in a two-plasmid system (5, 10). The advantages of using YF 17D as a live vector include (i) its established safety over a period of >60 years, during which over 350 million doses have been administered to humans; (ii) long duration of immunity after a single dose; and (iii) rapid onset of immunity within a few days after inoculation. The ChimeriVax technology obviates many of the problems of antivector immunity seen with live vectors, since the envelope genes are removed and replaced by the genes of the vaccine target. The chimeric vaccine virus causes an active infection in the recipient. Since the cytokine milieu and innate immune response are similar to those in natural infection, the antigenic mass expands in the host, and properly folded conformational epitopes are processed efficiently, the adaptive immune response is robust, and memory is established. The prM and E proteins derived from the target flavivirus contain the critical antigens for protective humoral and cellular immunity (27, 28). The immune response to infection with the vaccine virus, including all neutralizing antibodies, is directed principally at the target virus (DEN). The NS proteins of the YF 17D vector may also elicit cytotoxic T-lymphocyte responses and nonneutralizing antibodies against intracellular chimeric virus, but in pre-

TABLE 6. Neutralizing antibody titers in rhesus monkeys after secondary immunization (total dose, 5.4 $\log_{10}$ PFU) with ChimeriVax-DEN1–4 tetravalent vaccine

	Primary immunization [strain] (dose, $\log_{10}$ PFU)	50% neutralizing titer by indicated post-secondary immunization day vs:							
		YF-DEN1		YF-DEN2		YF-DEN3		YF-DEN4	
		180	210	180	210	180	210	180	210
T340	YF/DEN1 (4.3)	40	10,240	<10[a]	2,560	<10	1,280	<10	640
T791		160	10,240	<10	1,280	<10	640	<10	640
T744		640	2,560	20	320	10	320	<10	160
GMT[a] *(SD)*		160 (317)	6,451 (4,432)	3 (11)	1,016 (1,124)	2 (5)	640 (489)	1 (0)	403 (277)
T747	YF/DEN3 (3.6)	<10	640	<10	5,120	<10	5,120	<10	640
T383		80	1,280	40	640	640	1,280	20	80
T144		10	640	40	1,280	160	2,560	<10	160
GMT (SD)		9 (43)	806 (369)	12 (22)	1,613 (2,423)	47 (333)	2,560 (1,955)	3 (11)	207 (303)
T230	YF/DEN4 (3.8)	<10	40	<10	1,280	<10	160	<10	160
T260		10	160	20	640	40	640	2,560	2,560
T548		<10	160	<10	1,280	<10	320	320	1,280
GMT (SD)		2 (5)	101 (69)	3 (11)	1,016 (369)	3 (22)	320 (244)	94 (1,394)	806 (1,201)
T401	YF/DEN1–4 (5.3)	320	640	5,120	5,120	80	640	80	160
V744		640	640	1,280	1,280	1,280	640	1,280	2,560
BA28		80	1,280	2,560	2,560	160	160	160	640
T805		40	640	80	1,280	20	640	10	320
T354		160	640	640	1,280	80	640	40	320
T036		80	320	640	1,280	160	320	20	80
GMT (SD)		142 (229)	640 (315)	905 (1,870)	1,810 (1,550)	127 (485)	452 (213)	71 (500)	359 (941)
T725	WT D1 [PUO359] (3.9)	5,120	5,120	80	640	160	1,280	80	320
T613		5,120	5,120	320	640	640	1,280	80	320
T547		10,240	20,480	640	5,120	640	5,120	160	2,560
GMT (SD)		6,451 (2,956)	8,127 (8,868)	254 (281)	1,280 (2,586)	403 (277)	2,032 (2,217)	101 (46)	640 (1,293)
T502	WT D3 [PaH881/88] (5.2)	40	1,280	80	2,560	640	10,240	40	5,120
T600		80	1,280	80	2,560	2,560	10,240	40	2,560
T602		80	640	80	1,280	1,280	5,120	40	320
GMT (SD)		63 (23)	1,016 (369)	80 (0)	2,031 (739)	1,280 (978)	8,127 (2,956)	40 (0)	1,613 (2,402)
T264	WT D4 [1228] (3.8)	40	1,280	40	2,560	20	1,280	2,560	2,560
T529		10	320	20	640	40	320	2,560	1,280
T427		20	640	20	2,560	40	1,280	5,120	1,280
GMT (SD)		20 (15)	640 (489)	25 (11)	1,613 (1,108)	32 (11)	806 (554)	3,225 (1,478)	1,613 (739)

[a] For calculation of GMT, titers below <10 were considered 1.

TABLE 7. Viremia after YF/DEN1-4 tetravalent immunization (total dose, 5.4 $\log_{10}$ PFU) in YF-immune and nonimmune monkeys

YF immune	Monkey	Viremia ($\log_{10}$ PFU/ml) by post-secondary immunization day:											Mean		Serotype isolated[e] (day[s])
		1	2	3	4	5	6	7	8	9	10	11[f]	Peak titer[b] (SD)	Duration[c] (SD)	
Yes	BA29	—[a]	2.0	1.4	—	—	—	—	—	—	—	—	1.3 (0.9)	1.5 (0.7)	1, 2 (2–3)
	V576	—	—	—	—	—	—	—	—	—	—	—			
	V202	—	—	0.7	—	—	—	—	—	—	—	—			2 (3)
No	BF33	1.0	2.5	2.2	1.4	—	—	—	—	—	—	—	2.4 (0.2)	4.7 (1.5)	2 (1–4)
	BF29	—	2.3	2.5	1.9	—	—	—	—	—	—	—			2 (2–4), 3 (3)
	BJ43	1.4	2.1	2.1	1.4	—	—	—	—	—	1.4	2.2			2 (1–4), 4 (10–11)
	BD24	1.4	2.6	2.0	1.4	—	1.4	—	—	0.7	—	—			2 (3, 4, 6, 9)
	BB88	1.0	2.5	2.4	1.9	1.0	1.0	—	—	—	—	—			2 (1, 2, 4, 6)
	BG30	—	2.2	2.1	1.7	—	—	—	—	—	—	—			2 (2–4)
P[d]													0.007	0.007	

[a] —, <0.7 $\log_{10}$ PFU/ml.).
[b] $\log_{10}$ PFU per milliliter
[c] Days.
[d] Peak titers and duration of viremia in YF-immune group were compared to values from non immune group by *t* test (JPM version 4.0.2).
[e] Determined by RT-PCR followed by *Hin*dIII digestion.
[f] No viremia was detected after this day.

liminary studies in monkeys, these responses appeared not to provide cross-protection (10). The NS genes responsible for intracellular replication are derived from YF 17D and play an important role in attenuation (50). In the case of DEN2, it is clear that the envelope protein sequence is not necessarily linked to a virulence phenotype, since some attenuated live DEN2 vaccine candidates have WT envelope sequences (2). Hence, we predicted that YF/DEN2 chimeras with prME genes donated by WT DEN2 strains would be attenuated for nonhuman primates. This has proven to be the case (10). In addition, insertion of structural genes of WT DEN1 16007, but not its attenuated vaccine variant PDK-13, into the backbone of DEN2 PDK-53 produced a potential attenuated vaccine candidate for DEN1 virus (20).

All candidate YF/DEN chimeras against DEN serotypes 1 to 4 were constructed using gene donors (prME) from low-passage-number human isolates of DEN and were sequenced at early passages to establish consensus sequences. Sequencing of chimeras at Vero cell P3 or P4 posttransfection revealed some nucleotide and amino acid differences compared to their parent strains (Table 1). Insertion of a *Nar*I restriction site at the E/NS1 junction resulted in nucleotide changes at positions 1978 to 1980 (in DEN1, DEN2, and DEN4 chimeras) 1972 to

TABLE 8. Immunogenicity of YF/DEN1-4 tetravalent vaccine in YF-immune and nonimmune rhesus monkeys

YF immune	Monkey[b]	Titer on day 30 post-secondary immunization by:			
		DEN1	DEN2	DEN3	DEN4
Yes[a]	BA29	160	2,560	640	80
	V576	320	640	320	80
	V202	320	640	160	80
GMT (SD)		254 (92)	1,016 (1,108)	350 (244)	80 (0)
No	BF33	320	10,240	20	80
	BF29	160	2,560	40	80
	BJ43	160	640	20	160
	BD24	160	640	20	20
	BB88	160	640	40	40
	BG30	160	640	160	80
GMT (SD)		180 (65)	1,280 (3,840)	36 (55)	63 (48)
P[c]		0.17	0.60	0.013	0.91

[a] Refer to Table 3 for PRNT against YF.
[b] The PRNT against DEN1-4 was <10 at the time of secondary immunization.
[c] Titers of sera from YF-immune monkeys were compared to those of nonimmune monkeys by *t* test (JPM version 4.0.2).

TABLE 9. Immunogenicity of YF-VAX (5.3 $\log_{10}$ PFU) in YF/DEN1-4 tetravalent vaccine (one or two doses)-immune and nonimmune rhesus monkeys

YF/DEN1-4 immune	Group	Monkey	Mean viremia[a]		50% PRNT[b]
			Peak titer ($\log_{10}$ PFU/ml)	Duration (days)	
Yes (2 doses)	4	T036	—[c]	0	1,280
		T401	—	0	2,560
		V744	—	0	10,240
		BA28	—	0	5,120
GMT (SD)					3,620 (3,962)
Yes (1 dose)	9	BF33	1	1	5,120
		BF29	1	1	1,280
		B143	—	0	1,280
		BD24	—	0	1,280
		BB88	—	0	2,560
		BG30	—	0	1,280
GMT (SD)					1,810 (1,550)
No	10	CN82	2.3	3	2,560
		CN84	2.1	4	5,120
		CN81	2.4	4	5,120
		CN85	—	0	10,240
GMT (SD)					5,120 (3,221)
P			0.011[d]	0.006[d]	0.16[d], 0.04,[e] 0.7[f]

[a] Measured from days 1 to 12 post-YF-VAX immunization.
[b] Measured 30 days post-YF-VAX immunization in a plaque assay on Vero cells.
[c] —, No virus was detected.
[d] Compared across groups using one-way ANOVA test (JPM version 4.0.2).
[e] Between groups 9 and 10.
[f] Between groups 4 and 10.

1974 (in DEN3 chimeras), 1983 (in DEN1 and DEN4 chimeras), and 1977 (in DEN3 chimeras). These changes resulted in amino acid changes from Q to G at E494 of DEN1, DEN2, and DEN4 chimeras and at E492 of DEN3 chimeras. Additionally, there were five nucleotide changes in DEN1 and DEN2 chimeras, resulting in two amino acid substitutions (H to R at M39 and K to R at E204) in DEN1 and one substitution (I to V at E484) in DEN2 chimeras (10). The mutation leading to the I484V change, which was present as early as P1 posttransfection, was most likely present in the plasmids, since another chimeric DEN2 (MON310) previously constructed from these plasmids revealed the same substitution (9). Chimeric DEN3 revealed 13 nucleotide changes and 1 amino acid change (A to V at E489) compared to its parent DEN3 strain PaH881/88. Chimeric DEN4 revealed the highest number of amino acid substitutions compared to the parental DEN4 strain 1228 (seven nucleotide changes and four amino acid substitutions) (Table 1). Amino acid substitutions at E484, E489, E492, and E494 of chimeras, which are within the signal sequences for NS1 protein and located within the transmembrane TM2 domain of the E proteins (1), are less likely to affect the immunogenicity of chimeras. A mutation (M to V) at E477 within the TM2 of chimeric D2/D1 E protein was shown not to adversely affect the immunogenicity of this chimera in mice (20). Mutations in M proteins may not have a negative impact on the immunogenicity of chimeras, because the M proteins of mature flavivirions are believed to be masked by the E dimers and therefore may not be exposed to the host immune response. However, immature virus subpopulations containing uncleaved prM proteins are probably exposed on the surface of virions and may induce neutralizing and/or protective antibodies against prM or M protein. There have been some reports of prM-specific MAbs against DEN3, DEN4 (25), or Langat virus (21) that were protective in mice. By using synthetic peptides, the binding site of a protective MAb directed to prM proteins of DEN2 was identified to be within residues 40 to 49 of the M protein (7). It remains to be seen if an H-to-R change at M39 of chimeric DEN1 and an A-to-T change at M43 of chimeric DEN4 affect the immunogenicity of the viruses for mammalian hosts. Mutations observed in E proteins of chimeric DEN1 (K to R at E204) or chimeric DEN4 (V to I at E4, L to F at E56, and H to Y at E437) could affect the immunogenicity of these chimeras by alteration of the native conformation of neutralizing epitopes.

All chimeric viruses grew to peak titers of $\geq$7.5 $\log_{10}$ PFU/ml in Vero cells with the exception of chimeric DEN3. The reason for the slower growth of YF/DEN3 than of other chimeras might be due to the E489 mutation (Table 1). We recently reconstructed this virus without mutation and found that its growth rate improved 10-fold (data not shown).

The neurovirulence of chimeric viruses was assessed in 3- to 4-week-old outbred ICR mice by i.c. inoculation. This test was performed to ensure that the neurovirulence of chimeras does not exceed that of the parental YF-VAX, which is associated with rare postvaccinal encephalitis adverse events in humans (37). In contrast to YF-VAX, but similar to chimeric YF/DEN2 (10) and WT parent strains, DEN1, DEN3, and DEN4 chimeras were avirulent for 3- to 4-week-old outbred ICR mice inoculated with high doses (5 $\log_{10}$ PFU) by the i.c. route. We recently determined the i.c. 50% lethal dose (LD_{50}) of a premaster seed of chimeric YF/DEN2 viruses in 5- and 9-day-old suckling mice and compared it to that of YF-VAX. The LD_{50}s for chimeric DEN2 (0.76 and 3.3 $\log_{10}$ PFU for 5- and 9-day-old mice, respectively) were significantly higher than those for YF-VAX (-0.13 and <0.5 $\log_{10}$ PFU). The average survival time for chimeric YF/DEN2 was also higher than that for YF-VAX at all doses. Once premaster seeds for other chimeras are prepared, their suckling mouse i.c. LD_{50}s will be compared to that of YF-VAX. These data provide a high degree of confidence that the YF/DEN chimeric vaccines will be safer than YF-VAX with respect to neurotropism.

The pathogenesis of DEN fever in humans appears to be related to direct viral injury to extraneural tissues and cytokine release (31, 40). There is no animal disease model of dengue fever. However viremia in nonhuman primates reflecting extraneural replication of the virus generally reflects virulence for humans (47, 52, 57). In DEN-infected humans, higher viremia is associated with a more severe form of disease (DHF) (56). To assess the safety of YF/DEN chimeras, we therefore determined magnitude of viremia induced by test vaccines. The virus peak titers in sera of monkeys immunized with chimeras were compared to those of WT parent DEN or attenuated YF-VAX. All monkeys became viremic. However, the mean peak titers of viremia in monkeys inoculated with a monovalent or tetravalent chimeric virus (ranging from 0.7 to 1.5 $\log_{10}$ PFU/ml) were similar to that provoked by YF-VAX (1.9 $\log_{10}$ PFU/ml) (P values for peak titers and duration of viremia were 0.17 and 0.29 respectively) and significantly lower than their parent WT viruses (2.2 to 3.0 $\log_{10}$ PFU/ml) ($P = 0.003$). It is possible that more pathogenic genotypes of DEN, which are associated with severe forms of dengue infections (30, 44, 48), or DEN adapted to monkeys by serial passage would have produced even higher viremia than the current parental WT viruses (donors of prME genes in chimeras) which were isolated from humans with classical dengue fever. Interestingly, when peak viremias of YF/DEN chimeras were compared with those of their parental WT viruses (pairwise, using t test), YF/DEN1 ($P = 0.011$) and YF/DEN3 ($P = 0.016$) were significantly less viremic than their parental viruses but not YF/DEN4 ($P = 0.24$). The mean duration of viremia (1 to 3.3 days for chimeric viruses, 2.7 to 3.3 days for WT, and 2.3 days for YF-VAX) did not differ significantly across groups ($P = 0.18$) or between chimeric and parental viruses ($P = 0.18$ for YF/DEN1 and WT DEN1, $P = 0.07$ for YF/DEN3 and WT DEN3, and $P = 0.8$ for YF/DEN4 and WT DEN4). No association has been found between severity of disease and duration of viremia after DEN infection in humans (56). As predicted, no correlate between mouse neurovirulence and monkey viremia was observed; both chimeric and WT viruses were attenuated for 3- to 4-week old mice, but WT viruses induced significantly higher viremia in monkeys. In contrast, YF-VAX, which has been used for >60 years with extremely low incidence of adverse effects, was neurovirulent for mice but safe (attenuated) in monkeys (produced a low degree of asymptomatic viremia similar to that of chimeric viruses).

The neutralizing antibody titers were measured 30 and 79 days postimmunization (Table 3). All but two monkeys (one in the chimeric DEN3 group and one in the chimeric DEN4 group) that received the monovalent vaccines seroconverted. All six monkeys that received one dose of tetravalent vaccine

seroconverted to all four DEN serotypes except for one animal that did not seroconvert to DEN4. Monkeys immunized with monovalent chimeric viruses developed homologous neutralizing antibodies, but with titers lower than in animals given YF-VAX or WT DEN. These results indicate that the chimeric viruses are more attenuated than parental YF 17D. In the tetravalent group, antibody titers declined somewhat between days 31 and 79 for antibodies against DEN1 and DEN3 but not against DEN2 and DEN4. In a clinical study, out of 10 volunteers who received an empirically derived live attenuated tetravalent DEN vaccine, only one developed antibodies to all four serotypes, and similar to our observation, antibody levels measured 60 days postimmunization had declined in all subjects (24). All DEN chimeras except DEN1 could be detected in viremic monkeys inoculated with tetravalent vaccine by either serotype-specific monoclonal focus-forming assays or PCR-based restriction analysis. The most detectable virus by both methods in the tetravalent group was the YF/DEN2 chimera. Interestingly, the highest neutralizing titers in the tetravalent group were also directed against DEN2. DEN2 is the most important serotype in implication of DHF/DSS in secondary DEN infections. High levels of neutralizing antibodies to this virus after primary immunization with YF/DEN2 would thus provide immunity in vaccinees against the serotype most involved in DHF/DSS. However, in a widespread DHF, DEN3 has also frequently been implicated as a cause of DHF/DSS in Indonesia, Vietnam, and Thailand (13). Suboptimal immunity to one of the components of the tetravalent vaccine may theoretically increase the risk of developing DHF/DSS. Although several years' follow-up of volunteers given live DEN tetravalent vaccines in Thailand did not reveal any related DHF/DSS (N. Brahmarapravati and J. F. Saluzzo, personal communication), it is generally believed that an effective DEN vaccine should simultaneously induce high neutralizing antibody titers against all four serotypes. Because YF/DEN2 is more active or replicates earlier and may interfere with the other chimeric viruses included in a mixture of equal doses, it may be necessary to decrease the dose of the YF/DEN2 component in a tetravalent chimeric formulation. Similar conclusions were made following a study of live attenuated, empirically derived DEN vaccine in humans. In sera of 10 volunteers who received the DEN tetravalent vaccine (PDK derived), only one serotype (DEN3) could be detected. All 10 volunteers developed symptoms including fever and rash, but none developed dengue fever (24). It appeared from this study that the DEN3 component interfered with the other serotypes and was reactogenic.

To determine if the levels of antibody titers can be increased by a second dose, all monkeys were boosted by a tetravalent dose 6 months after primary immunization. Low levels of viremia were detected in monkeys previously immunized with monovalent chimeric viruses. No viremia was detected in any groups that received WT monovalent or tetravalent vaccines, demonstrating possible in vivo virus neutralization by heterologous (WT group) or homologous (tetravalent group) antibodies (Table 5). A serotype-specific anamnestic response was observed in all monkeys immunized with monovalent chimeric viruses, consistent with the principles of "original antigenic sin" well known in the case of sequential flavivirus infections (17, 19, 22, 29, 49). In addition, these monkeys developed broad neutralizing antibodies to the other three serotype viruses contained in the tetravalent booster vaccine. In acute and convalescent sera of patients with DHF or JE infection, a significant (≥4-fold) rise in DEN and JE neutralizing antibodies was observed. Similarly, sera of JE-infected patients with preexisting antibodies to YF showed a significant rise in YF and JE neutralizing antibodies (33, 58). These data indicate that cross-reactive antibodies with neutralizing activities are quite often induced in sequential flavivirus infection. The situation with chimeric viruses is unique, however, and unprecedented by previous studies of heterologous flavivirus serological interactions, since the nonstructural genes (of YF 17D) are identical in the priming and boosting virus. It is possible that the anamnestic response is driven in part by these carrier proteins, much as the response to polysaccharide conjugate vaccines is driven by prior inoculation of the carrier protein, e.g., diphtheria toxoid (8).

The two seronegative monkeys (T747 and T230 in groups 2 and 3, respectively) (Table 3) also developed neutralizing antibodies against all four serotpyes after the booster immunization with YF/DEN1-4 vaccine. Interestingly, the DEN1 chimera, which was never detected in serum after primary immunization, could be isolated along with chimeric DEN2 from monkey T747. It is possible that subneutralizing antibodies against DEN3 (50% neutralizing titer of <1:20 [Table 3]) in this monkey enhanced the replication of DEN1, a phenomenon known as antibody-dependent enhancement of infection (14–16, 26). No viremia (increase in replication of a serotype) was detected in any monkeys after the second dose, confirming that the simultaneous immunization including all four chimeric DEN serotypes has induced sufficient protective antibodies.

The issue of vector immunity is important for any live viral vaccine, because preexisting immunity to the vector in individuals may interfere with the efficacy of vaccination. The mechanism of antivector immunity in the case of two sequential chimeric viruses with different prME genes would involve cytotoxic T-lymphocyte responses against the shared (YF) NS3 protein and cytolytic antibodies against NS1. Attenuated recombinant poxviruses expressing JE genes (NYVAC-JEV) failed to induce neutralizing antibody responses in vaccinia virus-immune volunteers (23), whereas a recombinant poliovirus expressing the C-terminal half of chicken ovalbumin (Polio-Ova) produced similar antibody levels in both poliovirus-immune and nonimmune mice and protected them against lethal challenge with a tumor expressing the antigen (34).

Prior infection with one flavivirus may or may not modulate the viremic response to a heterologous flavivirus, depending in part on the level of antigenic relatedness. Thus, prior immunity to JE did not abrogate viremia following YF vaccination (53), whereas prior immunity to DEN (54) and certain other flaviviruses (18) cross-protected against YF. In the case of chimeric viruses, they share NS genes with YF 17D, and therefore cross-protection may be induced by humoral and cellular responses against infected cells early in infection, limiting antigen expression and subsequent immune response. Empirical studies were thus undertaken to determine whether preexisting immunity to YF 17D would interfere with YF/DEN vaccination. YF-immune and nonimmune monkeys were inoculated with tetravalent YF/DEN vaccine, whereas tetravalent immune and nonimmune monkeys were inoculated with YF-VAX. The interval between the two vaccines was 6 months.

Although the magnitude and duration of viremia in YF-immune monkeys were significantly lower than in nonimmune monkeys ($P = 0.007$ for both magnitude and duration of viremia) (Table 7), there was no statistically significant difference in titers of neutralizing antibodies within the two groups. The only exception was with YF/DEN3; neutralizing titers against the YF/DEN3 chimera (GMT = 350) in YF-immune monkeys was significantly higher than in nonimmune animals (GMT = 36) ($P = 0.013$) (Table 8). Similar enhancement has been reported in YF-immune subjects who received a live attenuated DEN2 (51) or a live attenuated chimeric YF/JE vaccine (Monath, unpublished). When chimeric tetravalent DEN-immune monkeys were inoculated with YF-VAX, no viremia was detected in any of monkeys previously immunized with two doses of tetravalent DEN vaccine, whereas a low level of viremia was detected in two monkeys that previously received only one dose of tetravalent DEN vaccine. YF-specific neutralizing antibody responses in nonimmune monkeys were significantly higher than those in monkeys previously immunized with one dose of tetravalent DEN vaccine ($P = 0.042$) but were similar to those in monkeys that previously received two doses of tetravalent DEN vaccine ($P = 0.72$) (Table 9). These data and those recently obtained from a YF/JE clinical trial in YF-immune and nonimmune volunteers (Monath, unpublished) indicate that antivector immunity will not be a significant factor limiting the practical utilization of chimeric vaccines and YF vaccine in humans.

In summary, all candidate YF/DEN chimeras against four serotypes (DEN1 to DEN4) were constructed using gene donors from low-passage-number human isolates of DEN. All chimeras replicated to high titers (6.3 to 8.0 $\log_{10}$ PFU/ml) in cells acceptable for good manufacturing practices production and were nonneurovirulent for 4-week-old ICR mice. In studies in nonhuman primates, viremia was lower than for parental WT strains and similar to viremia observed in controls given YF-VAX. Neutralizing antibodies against all four serotypes were elicited in almost all animals after a single dose of tetravalent vaccine, but titers were lower than for YF/DEN2. The titers of antibodies against all serotypes were increased when animals were boosted with a second dose of tetravalent vaccine. Despite suppression of viremia in YF-immune monkeys that received the tetravalent vaccine or tetravalent immune monkeys that received YF-VAX, no statistically significant differences were observed in magnitude of immune responses between immune and nonimmune monkeys. Optimization of the vaccine candidates and dose formulations to elicit high antibody titers may require genetic modifications of the DEN prME sequences as well as dose adjustment of the tetravalent formulation.

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