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Safety and Efficacy of Chimeric Yellow Fever-Dengue Virus Tetravalent Vaccine Formulations in Nonhuman Primates

F. Guirakhoo,[1]* K. Pugachev,[1] Z. Zhang,[1] G. Myers,[1] I. Levenbook,[2] K. Draper,[3] J. Lang,[4] S. Ocran,[1] F. Mitchell,[1] M. Parsons,[1] N. Brown,[1] S. Brandler,[1] C. Fournier,[4] B. Barrere,[4] F. Rizvi,[4] A. Travassos,[5] R. Nichols,[1] D. Trent,[1] and T. Monath[1]

Acambis, Inc., Cambridge, Massachusetts 02139[1]; Northbrook, Illinois 60062[2]; Sierra Division, Discovery and Development Services, Charles River Laboratories, Inc., Sparks, Nevada 89431[3]; Aventis Pasteur, Marcy-L'Etoile F-69280, France[4]; University of Texas Medical Branch, Galveston, Texas 77555-0609[5]

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To construct chimeric YF/DEN viruses (ChimeriVax-DEN), the premembrane (prM) and envelope (E) genes of yellow fever (YF) 17D virus were replaced with those of each wild-type (WT) dengue (DEN) virus representing serotypes 1 to 4. ChimeriVax-DEN1–4 vaccine viruses were prepared by electroporation of Vero cells with RNA transcripts prepared from viral cDNA (F. Guirakhoo, J. Arroyo, K. V. Pugachev, C. Miller, Z.-X. Zhang, R. Weltzin, K. Georgakopoulos, J. Catalan, S. Ocran, K. Soike, M. Ratteree, and T. P. Monath, J. Virol. 75:7290–7304, 2001; F. Guirakhoo, K. Pugachev, J. Arroyo, C. Miller, Z.-X. Zhang, R. Weltzin, K. Georgakopoulos, J. Catalan, S. Ocran, K. Draper, and T. P. Monath, Virology 298:146–159, 2002). Progeny viruses were subjected to three rounds of plaque purifications to produce the Pre-Master Seed viruses at passage 7 (P7). Three further passages were carried out using U.S. current Good Manufacturing Practices (cGMP) to produce the Vaccine Lot (P10) viruses. Preclinical studies demonstrated that the vaccine candidates are replication competent and genetically stable and do not become more neurovirulent upon 20 passages in Vero cells. The safety of a tetravalent vaccine was determined and compared to that of YF-VAX in a formal monkey neurovirulence test. Brain lesions produced by the tetravalent ChimeriVax-DEN vaccine were significantly less severe than those observed with YF-VAX. The immunogenicity and protective efficacy of four different tetravalent formulations were evaluated in cynomolgus monkeys following a single-dose subcutaneous vaccination followed by a virulent virus challenge 6 months later. All monkeys developed low levels of viremia postimmunization, and all the monkeys that had received equal concentrations of either a high-dose (5,5,5,5) or a low-dose (3,3,3,3) formulation seroconverted against all four DEN virus serotypes. Twenty-two (92%) of 24 monkeys were protected as determined by lack of viremia post-challenge. This report is the first to demonstrate the safety of a recombinant DEN virus tetravalent vaccine in a formal neurovirulence test, as well as its protective efficacy in a monkey challenge model.

Dengue is a mosquito-borne flavivirus infection caused by four dengue (DEN) virus serotypes (types 1 to 4), which in recent years has become a major international public health concern (13). Dengue is found in tropical and subtropical regions around the world, predominantly in urban and periurban areas. Classic dengue fever is a self-limited infection in children and adults; it begins with high fever, headache, retro-orbital pain, and facial flushing. The acute phase is followed by myalgia, arthralgia, nausea, and rash. A potentially lethal complication of dengue, dengue hemorrhagic fever (DHF)/dengue shock syndrome (DSS), was first recognized during the 1950s and today is a major disease affecting children in Asia. The global impact of dengue has expanded due to discontinuation of mosquito control programs, increase in air travel, growth of urbanization, and deterioration of public health programs. The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia, and the western Pacific. In April 2002, the World Health Organization (WHO) estimated that at least 2,500 million people—two-fifths of the world's population—were at risk of contracting dengue and that the number of infections worldwide may reach 50 million cases per year (51). Recent reports indicate that this number may be an underestimate (12, 33, 34). Vaccination would be the most efficient approach to control the disease. With the availability of recombinant DNA (rDNA) technologies, efforts to produce an effective dengue vaccine have been intensified. One of the most promising approaches involves the chimerization of two flaviviruses to produce attenuated dengue vaccine candidates. In this approach, the envelope (E) and premembrane (prM) genes of a flavivirus such as yellow fever (YF) 17D virus, DEN2 virus, or DEN4 virus, are replaced with those of dengue vaccine candidates (DEN virus serotypes 1 to 4). Monovalent chimeric DEN viruses have proven to be safe and immunogenic in animal models (2, 4, 8, 16, 20, 49) and human subjects (9) but have not been tested as tetravalent vaccines containing all four serotypes. It is generally accepted that a successful dengue vaccine needs to immunize against all four DEN serotypes simultaneously. The incompletely immunized in-

* Corresponding author. Mailing address: Acambis, Inc., 38 Sidney St., Cambridge, MA 02139. Phone: (617) 761-4323. Fax: (617) 494-1741. E-mail: farshad.guirakhoo@acambis.com.

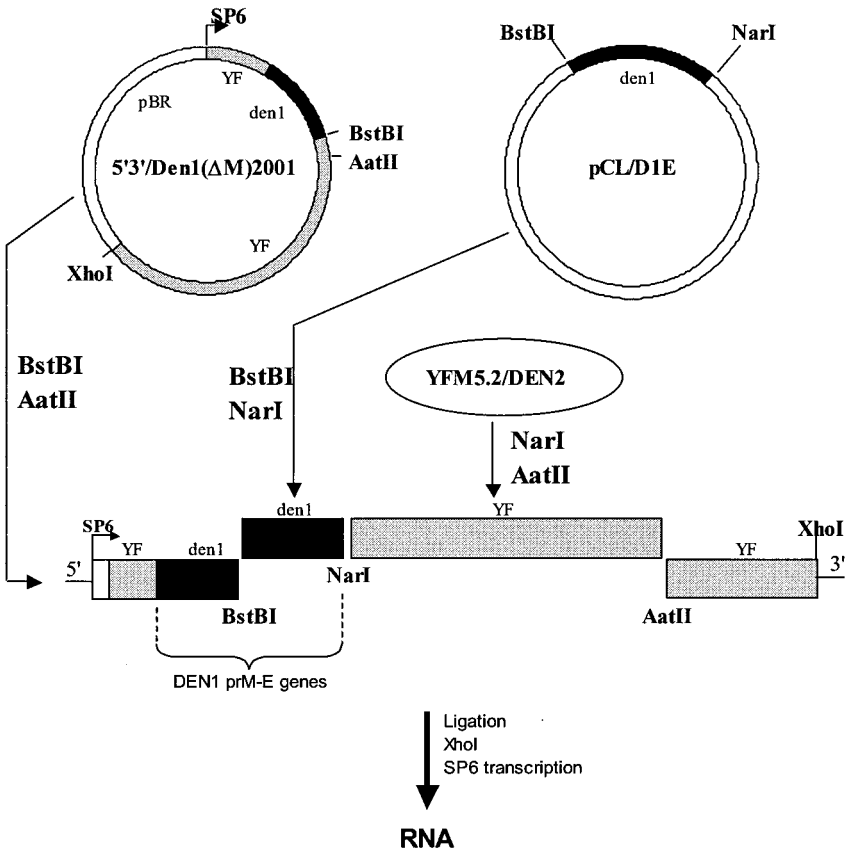

FIG. 1. Construction of ChimeriVax-DEN1 virus using a three-fragment ligation approach (see Materials and Methods).

dividual, or one in whom antibody titers wane, may be sensitized to a severe immunopathological disease (DHF/DSS) (18, 24, 46).

We have used the YF 17D virus vaccine virus as a vector for the construction of YF/DEN1 through YF/DEN4 chimeras (ChimeriVax-DEN1–4) and evaluated their safety and immunogenicity in mice and monkeys (14–16). A phase I clinical trial with monovalent ChimeriVax-DEN2 virus was completed recently, which showed an excellent safety and immunogenicity profile of the vaccine candidate (Acambis). All YF/DEN chimeras described previously were constructed using gene donors (prME) from low-passage human isolates of DEN viruses. These chimeras, which were generated by transfection of Vero cells with RNA transcripts and not subjected to plaque purifications, contained viruses with mutations in their genomes due to the quasispecies nature of RNA viruses. To manufacture vaccines for incorporation into a tetravalent formulation, a plaque purification strategy was adopted to produce a more homogeneous population of virus stocks that would be less likely to accumulate mutations during cell culture passages. When these viruses were analyzed after 20 passages in Vero cells, the uncloned viruses had accumulated more mutations than plaque purified chimeras (45).

This is the first report describing the safety of a recombinant chimeric tetravalent DEN virus vaccine in a formal monkey neurovirulence test and showing the protective efficacy of the vaccine in a monkey challenge model. Based on these results, a tetravalent dose and formulation were selected for use in a clinical trial.

MATERIALS AND METHODS

Generation of cDNA templates. The cDNA templates for in vitro transcription were prepared either by two-fragment (ChimeriVax-DEN2 and ChimeriVax-DEN4 [14–16]) or three-fragment (ChimeriVax-DEN3 [15]) ligation. For construction of ChimeriVax-DEN1, the original two-plasmid approach was found to be problematic because of the toxicity of the DEN1 virus-specific YFM5.2 plasmid for *Escherichia coli*. To resolve this problem, the original versions of this virus were obtained by an overlapping PCR step, which was not practical for vaccine manufacturing (14, 15). We therefore constructed three stably cloned plasmids to produce this chimera by the three-fragment ligation approach. First, the M-39 (H-to-R) mutation in the pYD1-5′3′ plasmid (14, 15) was eliminated by oligonucleotide-directed mutagenesis, resulting in plasmid 5′3′DEN1 (ΔM)2001 (Fig. 1). The BstBI-NarI fragment containing the C-terminal portion of the DEN1 virus E gene (normally present in YFM5.2 plasmids) was resynthesized by reverse transcription-PCR on virion RNA of DEN1 virus strain PUO359 and individually cloned in a modified low-copy-number vector, pCL1921, resulting in plasmid pCL/D1E. The absence of mutations in both plasmids was verified by sequencing. DNA template for in vitro transcription was prepared by three-fragment ligation (Fig. 1). The BstBI-NarI fragment of pCL/D1E plasmid and the Nar-AatII fragment from the YFM5.2/DEN2 plasmid (containing most of the YF virus-specific NS genes) (8, 16) were ligated with the large BstBI-AatII portion of the 5′3′DEN1(ΔM)2001 plasmid. Ligation products were linearized with XhoI and phenol-chloroform extracted, and RNA transcripts were synthesized in vitro using SP6 RNA polymerase (Epicentre).

Preparation of cloned ChimeriVax-DEN1–4 PMS viruses (non-GMP). Preparation of cloned Pre-Master-Seed (PMS) viruses for four DEN virus chimeras has been described previously (45). Briefly, Vero cells were electroporated with the corresponding RNA transcripts. The resulting passage 1 (P1) viruses were

harvested and amplified once to produce P2 viruses. The P2 viruses were used to initiate plaque purification. Three direct plaque purification steps (P3 to P5) were performed with Vero cells by using the standard agarose double-overlay method with Neutral Red staining. Following plaque purification, viruses were amplified twice to produce cloned PMS (P7) viruses. Based on the results of consensus sequencing, clones J, A, A, and B of ChimeriVax-DEN1, ChimeriVax-DEN2, ChimeriVax-DEN3, and ChimeriVax-DEN4, respectively, were selected as input PMS viruses for GMP-compliant manufacture of the vaccine stocks. The DEN1, DEN2, and DEN4 PMS viruses had no detectable mutations, whereas the DEN3 PMS virus had one silent nucleotide change (C to T) at nucleotide (nt) 6607.

Preparation of Master Seed, Working Seed, and Vaccine Lots of ChimeriVax-DEN1–4 viruses under cGMP. Master Seed (MS, P8) viruses were prepared at Molecular Medicine (La Jolla, Calif.) using GMP-compliant procedures. Vero cells were grown in MEME medium (supplemented with 2 mM L-glutamine, 1% minimal essential medium MEM nonessential amino acids, and 10% 0.1-μm-pore-size-filtered, gamma-irradiated fetal bovine serum (FBS) to near confluence in 10-layer Nunc cell factories (NCF). The cells were then infected with PMS (P7) virus at a multiplicity of infection of approximately 0.001 and incubated at 36 ± 2°C under 5% ± 2% CO_2 at 80% ± 5% relative humidity for 3 to 4 days. Virus-containing cell culture supernatant fluids were harvested when an early cytopathic effect was observed and processed by filtration to remove cell debris. The MS virus was formulated by adding FBS to a final concentration of 50%. The formulated materials were filtered through a sterile 0.22-μm-pore-size filter and stored at ≤−60°C. Working Seed (WS, P9) and Vaccine Lot (VL, P10) viruses were produced using GMP-compliant procedures by Aventis-Pasteur (Marcy L'Etoile, France). WS (P9) virus was manufactured, formulated, and filled in the same manner as MS (P8) virus. The manufacture of WS and VL viruses involved a biomass expansion phase in NCFs. For production of VL, the cells were washed twice with serum-free medium prior to virus infection and refed with serum-free medium to reduce the level of FBS in the final bulk. Downstream processing consisted of filtration to remove cell debris followed by digestion of nucleic acids by benzonase (15 UI/ml at 5 ± 3°C for 16 h), concentration by tangential-flow filtration (50 kDa), and diafiltration of the virus. VL viruses were stabilized by the addition of sorbitol to a final concentration of 10%.

Sequencing. Consensus sequencing of viruses was done as described previously (45). Briefly, virion RNAs were extracted from supernatants of infected cells by using TRI-Reagents LS (Molecular Research Center). RNAs were amplified with the Titan One-Tube reverse transcription-PCR kit (Roche), and cDNA fragments were purified using the QIAquick PCR purification kit or the QIAquick gel extraction kit (Qiagen) and sequenced using the CEQ DYE Terminator cycle-sequencing kit (Beckman). The sequencing reaction products were resolved with a CEQ2000 automated sequencer (Beckman Coulter) and analyzed by Sequencher 3.0 (GeneCodes).

Cells and viruses. LS-10 Vero cells were obtained from a qualified working cell bank at passage 137 from Aventis Pasteur and grown in MEME containing 10% FBS (HyClone, Ogden, Utah). In addition to ChimeriVax-DEN1–4 PMS, MS, WS, and VL, the following viruses were used: stocks of vaccine viruses (P10) for ChimeriVax-DEN1–4 were produced from MS virus stocks (P8) by two subsequent passages in Vero cells under non-GMP-compliant conditions; wild-type (WT) DEN virus stocks (DEN1 [West Pacific 74], DEN2 [S16803 PDK-10], DEN3 [CH53489], and DEN4 [341750]) were kindly provided by Kenneth Eckels (Walter Reed Army Institute of Research (WRAIR), Silver Spring, Md.); and YF-VAX (YF vaccube strain 17D) was purchased from Aventis Pasteur. All WT DEN viruses and YF-VAX were used without any further passages.

Potency and identity tests. Three different plaque assay techniques using Vero cells were used to measure the potency and identity of DEN viruses. The first was a standard plaque assay using agarose double overlay, where plaques were visualized by addition of Neutral Red to the plates. This technique was generally used to measure the potency of chimeras that produce visible plaques (16, 17); titers were expressed as PFU per milliliter. The second was an immunofocus-forming assay (IFF), using methyl cellulose instead of agarose, where plaques were visualized by the addition of DEN virus serotype-specific monoclonal antibodies and goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase. This technique was generally used for determination of the potency (Fig. 2) and identity of individual ChimeriVax-DEN serotype viruses in a mixture and for detection of WT DEN viruses that produce extremely small plaques in Vero cells (14). Titers were expressed as focus-forming units (FFU) per milliliter. The third was a modified IFF assay, where 6- or 12-well plates were replaced with 96-well plates. The titers in this assay, which is performed exclusively by Aventis Pasteur, were expressed as 50% tissue culture infectious dose per milliliter. Using selected samples, these three techniques produced comparable determinations of DEN virus potency.

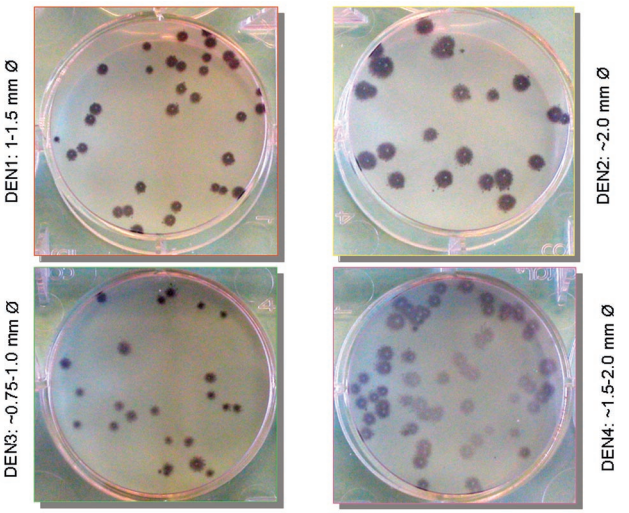

FIG. 2. Plaque morphology of ChimeriVax-DEN1–4 vaccine bulks identified by immunofocus-forming assay (see Materials and Methods).

Neutralization tests. Three different neutralization tests were performed on Vero cells. A plaque reduction method was used for measurement of immune response to homologous virus (chimeras). An immunofocus inhibition method was used to measure antibody to heterologous WT DEN virus challenge strains. In these tests, a constant virus input (~50 to 100 PFU) is neutralized by different serum dilutions and titers are expressed as the highest dilution of serum inhibiting 50% of the plaques ($PRNT_{50}$) (14–16, 36). For measurement of neutralizing antibody to YF 17D virus, a constant-serum varying-virus test was performed and the results were expressed as the $\log_{10}$ neutralization index (LNI) (35).

Animal studies. All studies were carried out using Institutional Animal Care and Use Committee-approved protocols and in accordance with the U.S. Department of Agriculture Animal Welfare Act (9 CFR parts 1 to 3), as described in the *Guide for Care and Use of Laboratory Animals* (41).

(i) Mice. The neurovirulence phenotypes of chimeras were assessed in suckling mice. Pregnant ICR (CD1) mice were purchased from Harlan (Indianapolis, Ind.). Suckling mice were pooled at the age of 2 to 3 days and randomly distributed to dams (9 to 13 mice/dam). The mice were inoculated at the age of 3 to 4 days by the intracerebral (i.c.) route with 0.02 ml of various passages of ChimeriVax-DEN1–4 viruses and observed for 21 days, and deaths were recorded. The virus dose administered to each group was determined by back titer determination.

(ii) Monkeys. Two studies were conducted with monkeys (Sierra Division of Discovery and Developments Services, Charles River Laboratories, Inc., Sparks, Nev.) to assess the safety, immunogenicity, and protective activity of ChimeriVax-DEN1–4 chimeras. Safety (neurovirulence) was assessed and compared to that of YF-VAX following i.c. inoculation, as prescribed by the WHO requirement for testing of YF virus vaccines (50) (experiment 1). Viremia and immunogenicity/protection were measured following subcutaneous (s.c.) inoculation with four different tetravalent formulations and challenge with WT DEN viruses (experiment 2).

(iii) Experiment 1. Twenty-two (11 male and 11 female) experimentally naive, flavivirus-seronegative cynomolgus monkeys (2.0 to 4.2 years of age and weighing 3.2 to 3.8 kg for males; 2.1 to 4.6 years of age and weighing 2.2 to 3.0 kg for females [day 1 values]) were assigned to two treatment groups ($n = 11$ for each group). The vaccine formulation (0.25 ml), consisting of approximately 5 $\log_{10}$ PFU of each cGMP ChimeriVax-DEN1–4 viruses (5.6 $\log_{10}$ PFU total, stabilized in 10% sorbitol solution) or reference article, YF-VAX (4.7 $\log_{10}$ PFU), was inoculated into the frontal lobe of the cerebral cortex. Animals were observed for 30 days postdose and then euthanized and necropsied. During the observation period, the monkeys were evaluated for clinical signs (twice daily), changes in food consumption (once daily), body weight (approximately weekly), serum chemistry, and hematology parameters (predose on day 1 and then on days 3, 5, 7, 15, and 31). Clinical signs were scored as specified by the WHO protocol (36, 50). Blood samples were collected on days 1 (predose) and 2 to 11 for viremia analysis and on days 1 and 31 for DEN1 through DEN4 virus antibody titer determination. At necropsy, gross pathological findings were recorded and tissues were preserved for histopathological examination. A selected subset of

tissues was evaluated by a board-certified pathologist (liver, spleen, heart, kidneys, and adrenal glands) or Inessa Levenbook (brain and spinal cord). Lesions in the meninges and the brain/spinal cord matter were scored using a scale of 0 to 2, on the basis of the following observations: grade 0, no visible lesions; grade 1 (minimal), one to three small and/or one large infiltrate, mostly perivascular; a few small foci of more diffuse infiltration, unconnected with blood vessels; grade 2 (mild), more than three small and/or two or more large perivascular infiltrates; several foci of cellular infiltration, unconnected with blood vessels (some neurons may be involved in these foci of inflammation). The degree of neurovirulence was estimated for the target and discriminator areas, as described previously (26, 36, 50). For cynomolgus monkeys, the substantia nigra and cervical and lumbar enlargements of the spinal cord represent the target formations whereas basal ganglia and thalamic nuclei are considered discriminator areas. Individual and group mean lesion scores for the target and discriminator areas were calculated both separately and as a combined score. Statistical analyses of lesion scores were performed by using a nonparametric Kruskal-Wallis test for comparison between groups.

(iv) Experiment 2. Twenty-four male and female monkeys were assigned to four treatment groups (six monkeys/group). Monkeys in each group received a single 0.5-ml s.c. dose of each of four tetravalent formulations (5,5,5,5 [group 1], 3,5,5,3 [group 2], 5,5,5,3 [group 3], and 3,3,3,3 [group 4] $\log_{10}$ $TCID_{50}$ of ChimeriVax-DEN1, ChimeriVax-DEN2, ChimeriVax-DEN3, and ChimeriVax-DEN4 viruses, respectively). Monkeys were evaluated for changes in clinical signs and body weight. Sera for virus and antibody titer determinations were collected at various time points. Viremia was measured on days 2 to 11. First, as a safety test, total viremia was measured to determine whether viremia levels were within acceptable limits according to the WHO guidelines (36, 50) (i.e., <500 mouse 50% i.c. lethal dose [LD] units for any individual monkey, estimated to equal 4.3 $\log_{10}$ Vero cell PFU/ml for YF-VAX). Next, titers of individual ChimeriVax-DEN viruses in sera were measured using a serotype-specific immunofocus assay on Vero cells (14). Neutralizing-antibody titers ($PRNT_{50}$) were measured in heat-inactivated sera (without addition of complement) obtained on days 1 (preimmune), 31, 121, 180 (prechallenge), and 210 (1 month postchallenge) against homologous or heterologous (WT DEN1 to DEN4 viruses, days 31, 180, and 210) viruses as described previously (14). To assess protective immunity, the monkeys were rerandomized into four new groups and challenged with WT DEN virus strains 6 months postimmunization. The control group consisted of 16 animals (4 monkeys per serotype) shown to be flavivirus naive by prescreening for presence of JE and DEN1 through DEN4 virus neutralizing antibodies by $PRNT_{50}$. Since the suitability of the challenge viruses had not been established previously in cynomolgus monkeys, 8 of 16 control monkeys (2 per serotype) were inoculated in advance with WT challenge strains to ensure that these viruses would induce acceptable levels of viremia in these monkeys. Viremia and neutralizing-antibody levels were measured postchallenge (5 $\log_{10}$ PFU/dose) to determine protection against each DEN virus serotype.

Statistical analysis. Differences in responses among groups or between two groups of animals were analyzed for significance using Fisher's exact test, two tailed for dichotomous variables and analysis of variance (ANOVA) for continuous variables; all analyses were performed using JMP software version 5.0. Statistical analyses of neuropathological lesion scores in experiment 1 were performed using a nonparametric Kruskal-Wallis test for comparison between groups.

RESULTS

Genomic stability. The genetic stability of PMS viruses passaged up to P20 has been described previously (45). Three-times plaque-purified DEN1, DEN2, and DEN4 PMS viruses had no amino acid substitutions at P7, and DEN3 PMS virus had a single silent nucleotide change (NS4a 6607 C to T). During subsequent GMP manufacture of the vaccines, mutations appeared in both the DEN1 and DEN2 chimeras. In the DEN1 chimera, nucleotide heterogeneity (containing WT A and mutant G) was observed at position 1590 as early as P8, resulting in the presence of viruses containing either K or R at the E204 residue. The mutant nucleotide was fixed at P9 (WS), remaining stable throughout vaccine production (P10) or further laboratory passages (to P20). This was an advantageous mutation, which reduced the neurovirulence of the DEN1 chimeric virus for infant mice and viscerotropism for monkeys (F. Guirakhoo, unpublished data). At P8 (MS), the DEN2 chimera contained some nucleotide heterogeneities in both the prM (T/G at nt 551) and E (G/T at nt 1730) genes, resulting in the presence of mixed amino acids L/V at prM24 and V/F at E251 positions. At P9, the prM24 mutation had already been established (resulting in an L-to-V substitution) whereas E251 was first observed as a complete mutant population at P10. There were no amino acid substitutions in the DEN3 or DEN4 chimeras following three passages of the P7 stock in Vero cells. On further Vero passages, starting with non-GMP P10 viruses, some nucleotide heterogeneities were observed within the NS4b gene of the DEN1 chimera (NS4b23 S/I and NS4b98 V/I at P20), whereas no further mutations were observed with DEN2 or DEN4 chimeras from P10 to P20 (Table 1). The DEN3 chimera showed a single amino acid substitution (L to F) at NS4b177 at P20. Interestingly, when DEN1 through DEN4 viruses were passaged to P20 starting with GMP P10 viruses, the NS4b 177 L-to-F mutation was detected in both DEN3 and DEN4 chimeras (Y. Girerd-Chambaz, personal communication). These mutations, which generally resulted in an increase in viral titers and plaque sizes, were most probably due to adaptations to Vero cells (45).

Plaque morphology. Extensive laboratory passages in Vero cells often resulted in adaptation, which consequently altered the plaque morphologies of chimeras. These phenotypic alterations could generally be explained by amino acid substitutions in the envelope genes. For example, DEN1 and DEN2 PMS viruses produced small plaques (~1 mm) in Vero cells at P7, which increased to ~2 to 3 mm during three further passages, as a result of mutations in the envelope genes (Table 1). In contrast, no change in the genome sequence of the DEN4 chimera was observed up to P20, despite a modest increase in its plaque size (from ~1 mm at P7 to ~2.0 mm at P20). To further search for the presence of virus mutants not detected by consensus sequencing, vaccine viruses were plaqued in Vero cells. The plaque morphology was determined after staining with DEN serotype-specific monoclonal antibodies. As shown in Fig. 2, only the DEN3 vaccine strain produced heterogeneous plaque sizes. Direct sequencing of small and large plaques from this vaccine revealed a minority population of mutant viruses (E202 K to R) that had not been identified by consensus sequencing, which has a limit of detection of about 10%. However, when a non-GMP version of DEN3 virus at P10 (vaccine passage level) that was produced by passage of the MS (P8) virus was subjected to consensus sequencing, both parent and mutant viruses could easily be detected (Table 1). Both small-plaque (E202K) and large-plaque (E202R) viruses retained their attenuated phenotype in the 4-day-old suckling-mouse model (data not shown).

Mouse neurovirulence. The neurovirulence of the DEN1 chimera for infant mice was significantly reduced from P8 to P10 ($P < 0001$ for all doses measured by the log-rank method). An increase in the 50% lethal dose from <2.1 (P7) to >5.1 $\log_{10}$ (P10) PFU/ml correlated with the appearance of the E204 K-to-R mutation (Table 1, experiment 1). Another clone of the DEN1 chimera (clone E) with a single E204 K-to-R mutation was also significantly less virulent than its parent WT chimera (Guirakhoo, unpublished). For the DEN2 and DEN3 chimeras, accumulations of mutations (prM24V and E251F in

TABLE 1. Neurovirulence of ChimeriVax-DEN1–4 viruses at various Vero passages in 4-day-old mice

Virus chimera	Expt	Passage[a]	Amino acid changes[b]	Dose (BT)[c]	Mortality (no. of dead/total no.)	LD_{50} (log_{10} PFU)
DEN1	1	P7 (PMS)	None	3.1, 2.1	10/11, 8/11	<2.1
		P8 (GMP MS)	E204K→R	2.6, 1.6	7/11, 8/11	<1.6
		P9	ND[d]	4.1, 3.1	2/11, 3/11	>4.1
		P10	E204K→R	5.1, 4.1, 3.1	1/13, 1/13, 0/12	>5.1
	2	P7	None	2.9, 1.9	10/10, 8/10	<1.9
		P20	E204K→R, NS4b23 (S→I), NS4b98(V→I)	3.8, 2.8	0/9, 0/9	>3.8
DEN2	1	P7 (PMS)	None	3.3, 2.3	11/11, 8/11	<2.3
		P8 (GMP MS)	prM24L→V, E251V→F	2.0, 1.0	11/11, 9/11	<1.0
		P9	ND	3.8, 2.8	11/12, 10/12	<2.8
		P10	prM24L→V, E251V→F	4.0, 3.0, 2.0	11/12, 10/13, 5/13	2.7
	2	P7 (PMS)	None	3.3, 2.3, 1.3	10/10, 8/10, 6/10	1.3
		P20	prM24L→V, E251V→F	3.6, 2.6, 1.6	7/9, 4/10, 2/9	3.5
DEN3	1	P7 (PMS)	None	3.6	4/12	>3.6
		P8 (GMP MS)	None	3.5	3/12	>3.5
		P9	ND	4.0, 3.0	0/13, 0/13	>4.0
		P10	E202K→R	3.7, 2.7	0/13, 0/13	>3.7
	2	P7 (PMS)	None	4.6, 3.6	2/10, 2/10	>4.6
		P20	NS4b177L→F	4.1, 3.1	0/10, 0/10	>4.1
DEN4	1	P7 (PMS)	None	2.3, 1.3	9/11, 6/11	2.2
		P8 (GMP MS)	None	2.5, 1.5	11/12, 9/11	<1.5
		P9	ND	2.4, 1.4	8/11, 1/12	1.9
		P10	None	4.0, 3.0, 2.0	12/13, 7/13, 4/13	2.4
	2	P7 (PMS)	None	2.6, 1.6, 0.6	4/11, 3/11, 0/11	>2.6
		P20	None	3.7, 2.7, 1.7	2/11, 1/11, 0/11	>3.7
YF-VAX	1	None	NA[d]	2.2	15/15	<2.2
	2	None	NA	2.2	9/9	<2.2

[a] P9 and P10 viruses were produced in the research laboratory by two passages of GMP MS (P8) in Vero cells.
[b] No silent mutation was found in any viruses except for DEN3 virus (a nucleotide change in NS4A6607 was found in all passages of DEN3 virus chimeras).
[c] Doses were determined by back titer determination (BT) of inocula.
[d] ND, not done; NA, not applicable.

DEN2 or heterogeneity in the E202 residue of DEN3) did not significantly alter their neurovirulence for infant mice. However, extensive passage in Vero cells (P20) seemed to decrease the neurovirulence of these viruses for mice, sometimes without an apparent genetic change (Table 1, experiment 2). All passages of the DEN1 through DEN4 virus chimeras were significantly less virulent ($P < 0001$, log-rank method) than their vector, YF 17D virus.

To demonstrate the absence of interference between chimeras administered as a tetravalent formulation, groups of 4-day-old infant mice were inoculated by the i.c. route with each of four chimeras as monovalent (2 log_{10} PFU) or tetravalent (2 log_{10} PFU of each serotype) formulations. A control group was inoculated with ~2 log_{10} PFU of YF-VAX. As expected, all nine mice in the YF-VAX group died, with an average survival time (AST) of 7.7 days. The mortality rate with monovalent chimeras was 44, 22, 0, and 33% with an AST of 13.7, 14, >21, and 13.3 days for DEN1, DEN2, DEN3, and DEN4 virus chimeras, respectively. The mortality rate (33%) and AST (14.7 days) in the tetravalent group did not differ significantly from that of the monovalent chimeras with the highest neurovirulence (i.e., DEN1, DEN2, and DEN4 viruses). This experiment demonstrated that the attenuated DEN3 virus chimera in a tetravalent mixture did not interfere with the replication of other chimeras in the brains of suckling mice.

Monkey studies. Two experiments were performed to address the safety (experiment 1) and efficacy (experiment 2) of tetravalent vaccine formulations in monkeys.

(i) Experiment 1. Evaluation of safety and neurovirulence following i.c. inoculation of tetravalent vaccine in monkeys. Twenty-two monkeys were divided into two groups ($n = 11$ for each group). The vaccine formulation (0.25 ml) consisting of approximately 5 log_{10} PFU of each cGMP ChimeriVax-DEN1–4 viruses (5.6 log_{10} PFU total) or reference article, YF-VAX (4.7 log_{10} PFU), was inoculated into the frontal lobe of the cerebral cortex. Following inoculation, there were no vaccine-related changes in body weight, serum chemistry, or hematology parameters. Neither vaccine produced gross pathological findings. Possible test and reference article-related clinical signs were limited to pupil dilation in 5 of 11 monkeys inoculated with YF-VAX (group 1) and in 2 of 11 monkeys inoculated with ChimeriVax-DEN tetravalent vaccine (group 2) between days 16 and 31.

(a) Viremia. The magnitude and duration of viremia are shown in Table 2. Of 11 monkeys inoculated with YF-VAX, 10 (91%) became viremic. The duration of viremia was 1–3 days, with peak titers ranging from 1.3 to 2.9 log_{10} PFU/ml. All 11 monkeys inoculated with tetravalent ChimeriVax-DEN became viremic. The duration of the viremia, as measured by total virus in the serum, was 4 to 9 days, with peak titers

TABLE 2. Mean peak viremia and number of viremic days following intracerebral inoculation of monkeys with tetravalent ChimeriVax-DEN or YF-VAX

Group (n = 11)	Virus	Mean peak titer[a] for all animals (viremic animals)	Mean duration[b] for all animals (viremic animals)
1	YF-VAX®[c]	2.3[e] (2.4)	2.1[f] (2.3)
2	ChimeriVax-DEN1–4[c]	2.9[e]	5.5[f]
	ChimeriVax-DEN1[d]	1.3 (2.3)	1.45 (1.8)
	ChimeriVax-DEN2[d]	1.1 (1.4)	0.64 (1.2)
	ChimeriVax-DEN3[d]	2.6	4.0
	ChimeriVax-DEN4[d]	2.8	4.4

[a] Log_{10} PFU per milliliter.
[b] Days.
[c] Total viremia measured by standard plaque assay using crystal violet-stained Vero cells.
[d] Serotype-specific viremia measured by IFF assay specific for each DEN virus serotype.
[e] $P < 0.0001$ (ANOVA), YF-VAX versus ChimeriVax-DEN1–4.
[f] $P = 0.0094$ (ANOVA), YF-VAX versus ChimeriVax-DEN1–4.

TABLE 3. Summary of histopathological evaluation of brain and spinal cord in monkeys inoculated by the i.c. route with tetravalent ChimeriVax-DEN vaccine or YF-VAX

Treatment group	No. of monkey	Lesion scores (mean ± SD)		
		Target areas	Discriminator areas	Combined
YF-VAX®	11	0.43 ± 0.36	0.35 ± 0.32	0.39 ± 0.32
Tetravalent ChimeriVax-DEN	11	0.05 ± 0.08	0.03 ± 0.06	0.04 ± 0.05
P value[a]		0.0023	0.0015	0.0018

[a] Nonparametric Wilcoxon/Kruskal-Wallis test (rank sums) was used for statistical comparison of groups.

ranging from 1.7 to 3.3 log_{10} PFU/ml. The mean duration of viremia for tetravalent ChimeriVax-DEN vaccine (5.5 days) was significantly longer than that of YF-VAX (2.09 days) (P = 0.0094, ANOVA). Although the mean peak total viremia (i.e., the combined titers of all four chimeras) titer for tetravalent ChimeriVax-DEN (2.9 log_{10}PFU/ml) was higher than the mean peak titer for the reference YF-VAX (2.3 log_{10} PFU/mL) ($P < 0.0001$, ANOVA) virus, the titers for both vaccines remained within acceptable group and individual monkey specifications, based on WHO requirements for the YF 17D vaccine (50).

All four DEN virus chimeras were detectable in the sera, with the serotype-specific mean peak titers and duration of viremia varying somewhat with the virus strain. DEN1 virus specific viremia was detected in the sera of 9 (82%) of 11 monkeys inoculated with tetravalent ChimeriVax-DEN vaccine. The duration of viremia was generally 2 to 3 days, with peak titers ranging from 1 to 2 log_{10} PFU/ml. The mean peak titer and duration of viremia were 1.3 log_{10} PFU/ml and 1.45 days, respectively (considering both viremic and nonviremic animals). DEN2 virus specific viremia was detected in the sera of 6 (55%) of 11 monkeys. The duration of viremia was generally 1 to 3 days, with peak titers ranging from 1 to 1.7 log_{10} PFU/ml. The mean peak titer and duration of viremia were 1.1 log_{10} PFU/ml and 0.64 days, respectively (considering both viremic and nonviremic animals). DEN3 virus specific viremia was detected in the sera of 11 (100%) of 11 monkeys. The duration of viremia was generally 4 to 7 days, with peak titers ranging from 1.5 to 3.1 log_{10} PFU/ml. The mean peak titer and duration of viremia were 2.6 log_{10} PFU/ml, and 4 days, respectively. DEN4 virus specific viremia was detected in the sera of 11 (100%) of 11 monkeys. The duration of viremia was generally 4 to 8 days, with peak titers ranging from 1 to 3.2 log_{10} PFU/ml. The mean peak titer and duration of viremia were 2.8 log_{10} PFU/ml, and 4.4 days, respectively.

(b) Neutralizing-antibody response. All monkeys seroconverted following i.c. inoculation with YF-VAX. On day 31, YF virus-specific neutralizing-antibody titers ranged from 2.07 to 3.88 in the LNI assay (an LNI of $\geq$0.7 log_{10} is generally considered seropositive). A small proportion (2 of 11, 2 of 11, 1 of 11, and 1 of 11) of the YF-VAX-treated monkeys showed cross-reactivity with heterologous DEN1, DEN2, DEN3, or DEN4, respectively, in a $PRNT_{50}$ assay on day 31. Such antibody cross-reactivity is not unexpected among flaviviruses. However, remote exposure of these monkeys to a heterologous flavivirus not detected by prestudy antibody screening cannot be ruled out.

All monkeys treated with tetravalent ChimeriVax-DEN vaccine seroconverted to each DEN serotype. Neutralizing-antibody titers ranged from 160 to 20,480, 40 to 2,560, 20 to 640, and 80 to 5,120 for DEN1, DEN2, DEN3, and DEN4 viruses, respectively, in the PRNT assay; no monkeys had cross-reacting antibodies to YF 17D virus in the LNI assay. The geometric mean titer (GMT) was 1,202, 640, 85, and 726 for DEN1, DEN2, DEN3, and DEN4 virus chimeras, respectively.

(c) Histopathology. Neither vaccine produced histopathological findings in nonneural tissues. In neural tissues, the neurovirulence of the tetravalent ChimeriVax-DEN vaccine was minimal. The target, discriminator, and combined scores for the group given tetravalent ChimeriVax-DEN vaccine were significantly lower than the scores for the group that received YF-VAX ($P < 0.05$). Vaccine-related lesions in the meninges and in the brain or spinal cord of monkeys from both groups were limited to minimal to mild inflammation. These lesions were observed in 10 (91%) of 11 monkeys treated with YF-VAX and in 6 (55%) of 11 monkeys inoculated with tetravalent ChimeriVax-DEN vaccine. Perivascular infiltrates consisted of mononuclear cells. Rare foci of microglial cell infiltration of blood vessels were noted but considered unrelated to vaccine administration. The lesions in monkeys vaccinated with tetravalent ChimeriVax-DEN vaccine were significantly less severe than those in monkeys vaccinated with YF-VAX vaccine. Lesions induced by tetravalent ChimeriVax-DEN vaccine did not exceed grade 1, but lesions caused by YF-VAX were scored 1 or 2 (Table 3).

(ii) Experiment 2. Immunogenicity and protective efficacy of tetravalent formulations after one s.c. dose in cynomolgus monkeys. Twenty-four male and female monkeys were assigned to four treatment groups (six monkeys/group). The monkeys in each group received a single 0.5-ml s.c. dose of each of four tetravalent formulations (5,5,5,5 [group 1], 3,5,5,3 [group 2], 5,5,5,3 [group 3], and 3,3,3,3 [group 4] log_{10} $TCID_{50}$ of ChimeriVax-DEN1, ChimeriVax-DEN2, ChimeriVax-DEN3, and ChimeriVax-DEN4 viruses, respectively). There were no vaccine-related clinical signs or changes in food consumption or body weight.

TABLE 4. Summary, total, and serotype-specific viremia in cynomolgus monkeys immunized s.c. with one dose of ChimeriVax-DEN1–4 GMP vaccine tetravalent formulations

Group	Dose (log_{10} PFU)	No. viremic/ total no.	Viremia[a]			
			Mean peak (log_{10} PFU/ml)		Mean duration (days)	
			Total	Serotype	Total	Serotype
1	5,5,5,5	6/6	2.6	D1 = 1.2 (1.5)[b] D2 = 1.1 (1.6)[b] D3 = 2.3 D4 = 1.9	4.0	D1 = 1.5 (1.8)[b] D2 = 0.8 (1.2)[b] D3 = 2.3 D4 = 3.7
2	3,5,5,3	6/6	2.4	D1 = 0 D2 = 1.5 (2.3)[b] D3 = 1.8 (2.6)[b] D4 = 0.9 (2.7)[b]	2.5	D1 = 0 D2 = 1.3 (2)[b] D3 = 3 (4.5)[b] D4 = 1.5 (4.5)[b]
3	5,5,5,3	6/6	2.1	D1 = 1.2 (1.8)[b] D2 = 1.4 (1.7)[b] D3 = 1.8 (2.6)[b] D4 = 1.4 (2.1)[b]	3.0	D1 = 1.5 (2.2)[b] D2 = 2 (2.4)[b] D3 = 2.2 (2.6)[b] D4 = 2.3 (2.8)[b]
4	3,3,3,3	6/6	2.5	D1 = 1.2 (1.9)[b] D2 = 1.9 D3 = 1.6 (2)[b] D4 = 2.5	5.5	D1 = 2 (3)[b] D2 = 3.8 D3 = 3.5 (4.2)[b] D4 = 5.3

[a] D1, ChimeriVax-DEN1; D2, ChimeriVax-DEN2; D3, ChimeriVax-DEN3; D4, ChimeriVax-DEN4.
[b] Viremic animals only.

(a) Viremia. All 24 monkeys became viremic (Table 4). The onset of viremia in monkeys inoculated with the high-dose formulation (group 1) occurred as early as day 2, and all six animals were viremic on day 3. No viremia was detected in any group 1 monkey after day 8 (level of detection, $\leq$1.7 log_{10} PFU/ml). The mean peak titer and duration of viremia for this group were 2.6 log_{10} PFU/ml and 4.0 days, respectively. In low-dose group animals (group 4), viremia was delayed until day 3. Only one of six animals showed borderline viremia of 1.7 log_{10} PFU/ml on this day. Six (100%) of six monkeys were viremic from day 6 until the last day of serum collection (day 11). The mean peak titer and duration of viremia were 2.5 log_{10} PFU/ml and 5.5 days, respectively. Viremia in groups 2 and 3 (where the doses of DEN1 and/or DEN4 virus chimeras were reduced) was observed from days 2 to 11, due to circulation of different serotypes on each day (Table 5). The lowest mean

TABLE 5. Identification of ChimeriVax-DEN serotypes in viremic monkeys

Group	Monkey	ChimeriVax-DEN serotypes detected by study day[a]:									
		2	3	4	5	6	7	8	9	10	11
1	F20967F	—[b]	3	—	4	—	—	—	—	—	—
	F21343M	4	1–4	3	1,3	4	3,4	—	—	—	—
	F21786F	1,4	1–4	1–4	3,4	4	4	—	—	—	—
	F213114F	4	1–4	3,4	3	—	—	—	—	—	—
	F21339M	—	1,3,4	4	—	—	—	—	—	—	—
	F21386F	—	1–4	1,3,4	4	4	4	—	4	—	—
2	F21501M	—	3	3	3	3	—	—	—	—	—
	F212117F	—	—	—	—	—	—	—	—	—	4
	F21355M	2,3,4	2,3,4	2,3	4	4	4	4	4	3,4	3
	F20977F	—	3	2,3	—	3	3	3	3	—	2
	F21534M	—	—	—	—	—	—	—	—	—	2
	F21565F	2,3	2,3	3	—	—	—	—	—	—	—
3	F21342M	1,2,3	1,2,3	1,3	—	—	—	—	4	4	—
	F212105F	1,2,3	1,2,3	3	—	—	4	—	4	4	—
	F21544M	—	—	—	—	—	—	3	—	3	4
	F21784F	—	1,2,3	2,3,4	—	—	—	—	—	—	—
	F21149M	—	—	—	—	—	—	—	—	2	—
	F21384F	1,4	1,2,3	2,3,4	3	1,2	2,4	4	2,4	4	4
4	F209108F	—	—	—	—	1–4	1,3,4	1,3,4	3,4	—	—
	F21311M	—	2,3	2,3,4	2,3,4	1,2,4	1,2,4	1,2,4	1–4	3,4	4
	F18172F	—	4	1–4	1–4	1–4	1–4	4	—	—	—
	F20788F	—	2	—	1,2,4	2	2	—	2	4	—
	F21522M	2	2,3,4	2	2,3,4	2,3,4	4	—	4	4	—
	F21570F	—	—	2,3	3	4	3,4	4	4	3,4	3,4

[a] Monkeys were immunized on day 1. 1, ChimeriVax-DEN1; 2, ChimeriVax-DEN2; 3, ChimeriVax-DEN3; 4, ChimeriVax-DEN4.
[b] —, <1.3 log_{10} PFU/ml.

TABLE 6. Neutralizing-antibody response ($PRNT_{50}$) in monkeys 1 month after immunization with four ChimeriVax-DEN1–4 tetravalent formulations[a]

Formulation	Monkey	$PRNT_{50}$ against:			
		DEN1	DEN2	DEN3	DEN4
5,5,5,5 (group 1)	F20967F	320	5,120	1,280	>2,560
	F21343M	40	80	40	640
	F21786F	160	1,280	320	1,280
	F213114F	40	160	320	640
	F21339M	160	1,280	320	>2,560
	F21386F	160	1,280	320	640
GMT[b]		113	718	285	1,140
3,3,3,3 (group 4)	F209108F	160	160	160	>2,560
	F21311M	40	40	160	1,280
	F18172F	320	20	320	640
	F20788F	80	320	2,560	640
	F21522M	80	80	80	1,280
	F21570F	20	10	2,560	>2,560
GMT		80	56	403	1,280
3,5,5,3 (group 2)	F21501M	80	1,280	640	640
	F212117F	320	<10	10	<10
	F21355M	40	80	2,560	>1,280
	F20977F	10	2,560	320	<10
	F21534M	40	40	320	<10
	F21565F	640	>10,240	640	>1,280
GMT		80	218	320	31
5,5,5,3 (group 3)	F21342M	320	320	10,240	1,280
	F212105F	80	20	1,280	160
	F21544M	160	<10	320	<10
	F21784F	2,560	160	5,120	>1,280
	F21149M	10	40	<10	<10
	F21384F	80	40	640	320
GMT		142	34	489	66

[a] All animals had a $PRNT_{50}$ of <10 prior to immunization. WT heterologous challenge viruses were received from WRAIR (see Materials and Methods).

[b] For calculation of GMTs, titers of <10 were assigned a value of 1 and titers with unknown end points were assigned as shown (e.g., a titer of >1,280 was considered as 1,280).

peak viremia (2.1 $\log_{10}$ PFU/ml) was identified in group 3, where animals had received a reduced dose of ChimeriVax-DEN4 virus. The shortest duration of viremia (2.5 days) was observed in group 2, where animals had received reduced doses of DEN1 and DEN4 virus chimeras.

Serotype-specific viremia was detected in serum samples obtained from days 2 to 11. ChimeriVax-DEN1–4 viruses could be detected in all four groups inoculated with different formulations (level of detection, 1.3 $\log_{10}$ PFU/ml). The following serotypes were detected in sera within groups 1 to 4 (Table 5).

In group 1, all four serotypes were detected in four (67%) of six monkeys on day 3. All animals became viremic to DEN3 and DEN4 virus chimeras. DEN3 and DEN4 viruses were found in the majority of monkeys up to day 7, but DEN2 and DEN1 virus chimeras were not detected beyond days 4 and 5, respectively, in any animal. DEN1 and DEN2 virus chimeras and DEN2 virus chimera could not be found in monkeys F20967 and F21339M, respectively. The highest peak viremia, which lasted for 6 days, was induced by the DEN4 virus chimera (in monkey F21786F). The mean peak viremia titers were 1.2, 1.1 (1.5 and 1.6 when only viremic animals were considered, respectively), 2.3, and 1.9 $\log_{10}$ PFU/ml for DEN1 to DEN4 viruses, respectively. The mean duration of viremia was 1.5, 0.8 (1.8 and 1.2 when only viremic animals were considered, respectively), 2.3, and 3.7 days for DEN1 to DEN4 viruses, respectively (Table 4).

In group 2, monkeys were immunized with 100-fold-lower doses of ChimeriVax-DEN1 and ChimeriVax-DEN4. No DEN1 virus chimera could be detected in any of the monkeys. Other chimeras were detected from days 2 to 11 (Table 5). Four (67%) of six monkeys became viremic to DEN2 and DEN3 viruses. Only two (33%) of six monkeys became viremic to DEN4 virus. The highest viremia (3.4 $\log_{10}$ PFU/ml), which lasted for 3 days, was caused by DEN3 virus in monkey F21565F. The longest duration of viremia was 8 days with DEN4 virus in monkey F21355M. The mean peak viremia titers were 0, 1.5, 1.8, and 0.9 (0, 2.3, 2.6, and 2.7 when only viremic animals were considered, respectively) $\log_{10}$PFU/ml for DEN1 to DEN4 viruses, respectively. The mean durations of viremia were 0, 1.3, 3, 1.5 (2, 4.5, and 4.5 days when only viremic animals were considered, respectively) for DEN1 to DEN4 viruses, respectively (Table 4).

In group 3, most of the monkeys showed early viremia with DEN1 to DEN3 viruses and a delay in viremia (replication) with the DEN4 virus chimera. Only DEN3 and DEN4 viruses could be detected in monkey F21544M, and only the DEN2 virus chimera could be detected in monkey F21149M (for 1 day [Table 5]). The highest peak viremia (3.1 $\log_{10}$ PFU/ml) was induced by DEN3 virus (monkey F21342M) and DEN4 virus (monkey F21384F). The duration of viremia in these animals was 3 and 7 days, respectively. The mean peak viremia titers were 1.2, 1.4, 1.8, 1.4 (1.8, 1.7, 2.2, and 2.1 when only viremic animals were considered, respectively) $\log_{10}$ PFU/ml for DEN1 to DEN4 viruses, respectively. The mean duration of viremia was 1.5, 2, 2.2, 2.3 (2.2, 2.4, 2.6, and 2.8 days when only viremic animals were considered, respectively) days for DEN1 to DEN4 viruses, respectively (Table 4).

In group 4, chimeric viruses could generally be detected before day 3 (the exception was monkey F21522M, in which the DEN2 virus chimera could be detected on day 2). In contrast to group 1, in which most viruses were detected on days 3 to 4 and no virus was detected beyond day 7, the majority of viruses in group 4 were detected between days 5 and 7 and the DEN3 and DEN4 virus chimeras could be detected through day 11 (Table 5). All monkeys became viremic to DEN4 virus, and four of six became viremic to DEN1, DEN2, and DEN3 viruses (DEN1 virus could not be found in monkeys F21522M and F21570F). The highest peak viremia (3.1 $\log_{10}$ PFU/ml) was induced by the DEN4 virus chimera (monkey F209108F) and lasted for 4 days. The longest duration of viremia (8 days) was also related to the DEN4 virus chimera (monkey F21311M), with a peak titer of 2.8 $\log_{10}$ PFU/ml. The mean peak viremia titers were 1.2 (1.9 when only viremic animals were considered), 1.9, 1.6 (2.0 when only viremic animals were considered), and 2.5 $\log_{10}$ PFU/ml for DEN1 to DEN4 viruses, respectively. The mean duration of viremia was 2 (3 when only viremic animals were considered), 3.8, 3.5 (4.2 when only viremic animals were considered), and 5.3 days for DEN1 to DEN4 viruses, respectively (Table 4).

(b) Neutralizing-antibody response. Sera obtained on day 1 (preimmunization) were negative ($PRNT_{50} < 10$) against four DEN virus serotypes. As shown in Table 6, all monkeys (six of six) in group 1 seroconverted to all four chimeric DEN viruses

TABLE 7. Protection of monkeys immunized with ChimeriVax-DEN1–4 tetravalent formulations and challenged with heterologous WT DEN1 virus[a]

Group	Monkey	Vaccine formulation	Viremia by day postchallenge:										$PRNT_{50}$	
			2	3	4	5	6	7	8	9	10	11	Prechallenge[c]	Postchallenge[d]
5	F20967F	5,5,5,5	0[b]	0	0	0	0	0	0	0	0	0	160	5,120
	F21501M	3,5,5,3	0	0	0	0	0	0	0	0	0	0	40	20,480
	F21784F	5,5,5,3	0	0	0	0	0	0	0	0	0	0	40	20,480
	F18172F	3,3,3,3	0	0	0	0	0	0	0	0	0	0	80	20,480
	F21149M	5,5,5,3	0	0	0	0	2.0	3.0	3.3	1.7	0	0	20	20,480
	F21570F	3,3,3,3	0	0	0	0	0	0	0	0	0	0	160	10,240
6	F20928M	None	0	1.0	1.7	2.7	3.0	2.8	0	0	0	0	<10	2,560
	F22673F	None	0	0	1.0	1.3	2.9	3.3	3.2	1.7	0	0	<10	1,280
	F21753M	None	0	0	2.0	2.7	2.9	3.4	3.4	1.7	1.0	0	<10	2,560
	F21143F	None	0	1.0	1.7	1.7	2.5	3.3	2.6	0	0	0	<10	>10,240

[a] All monkeys were challenged with 5 log units of DEN1 virus (West Pacific 74, nonattenuated), with the exception of monkeys F20928M and F22673F, which received 4 log units of challenge virus (pilot study).
[b] No virus could be detected when serum was tested in the plaque assay at undiluted, 1:2, or 1:10 dilutions; limit of detection, 1 $\log_{10}$ PFU/ml.
[c] 180 days postvaccination.
[d] 30 days postchallenge (day 210).

by day 31 postimmunization and remained seropositive until challenged with WT viruses 6 months later. The level of DEN virus-specific neutralizing antibodies (day 31) varied from 40 to 320 for DEN1, 80 to 5,120 for DEN2, 40 to 1,280 for DEN3, and 640 to >2,560 for DEN4 viruses. The GMTs on day 31 were 113, 718, 285, and 1,140 for DEN1 to DEN4 viruses, respectively. All monkeys in group 4 also seroconverted to all four chimeric DEN viruses by day 31 postimmunization. The level of DEN virus-specific neutralizing antibodies varied from 20 to 320 for DEN1 virus, 10 to 320 for DEN2 virus, 160 to 2,560 for DEN3 virus, and 640 to >2,560 for DEN4 virus. The GMTs on day 31 were 80, 57, 403, and 1,280 for the DEN1 to DEN4 viruses, respectively. Only three (50%) of six monkeys in the dose-adjusted formulation group 3 seroconverted to all four chimeric DEN viruses by day 31 postimmunization. Three monkeys (F212117F, F20977F, and F21534M) did not seroconvert to DEN4 virus. Interestingly, monkeys F20977F and F21534M, which were later challenged with WT DEN4 virus, had developed a low $PRNT_{50}$ of 20 and 40, respectively, prior to challenge (see below). The level of DEN-specific neutralizing antibodies varied from 10 to 640 for DEN1 virus, <10 to >10,240 for DEN2 virus, 10 to 2,560 for DEN3 virus, and <10 to >1,280 for DEN4 virus. The GMTs on day 31 were 80, 218, 320, and 32 for DEN1 to DEN4 viruses, respectively. Four of six monkeys in group 4 seroconverted to all four chimeric DEN viruses by day 31 postimmunization. One monkey (F21544M) did not seroconvert to DEN2 and DEN4 viruses, and one monkey (F21149 M) did not seroconvert to DEN3 and DEN4 viruses. The level of DEN virus-specific neutralizing antibodies varied from 10 to 2,560 for DEN1 virus, <10 to 320 for DEN2 virus, <10 to 10,240 for DEN3 virus, and <10 to >1,280 for DEN4 virus. The GMTs on day 31 were 142, 34, 489, and 66 for DEN1 to DEN4 viruses, respectively.

(c) Protection against challenge with heterologous WT DEN strains at 6 months post immunization. To assess protective immunity, 22 of 24 monkeys in the original groups 1 to 4 were randomized into four new groups 5, 7, 9, and 11, as shown in Tables 7 to 10. Two animals (F20977F and F21534) that did not seroconvert to DEN4 virus after primary vaccination were allocated to group 11 to be challenged with DEN4 virus. A control group of an additional 16 monkeys were randomly assigned to groups 6, 8, 10, and 12 (four animals per group) (Tables 7 to 10). Viremia (2 to 11 days) and

TABLE 8. Protection of monkeys immunized with different ChimeriVax-DEN1–4 tetravalent formulations and challenged with heterologous WT DEN2 virus[a]

Group	Monkey	Vaccine formulation	Viremia by day postchallenge:										$PRNT_{50}$	
			2	3	4	5	6	7	8	9	10	11	Prechallenge[c]	Postchallenge[d]
7	F212117F	3,5,5,3	0[b]	0	0	0	0	0	0	0	0	0	20	640
	F21342M	5,5,5,3	0	0	0	0	0	0	0	0	0	0	80	5,120
	F21786F	5,5,5,5	0	0	0	0	0	0	0	0	0	0	160	1,280
	F21384F	5,5,5,3	0	0	0	0	0	0	0	0	0	0	80	2,560
	F21544M	5,5,5,3	0	0	0	0	0	0	0	0	0	0	<10	1,280
	F21386F	5,5,5,5	0	0	0	0	0	0	0	0	0	0	40	640
8	F22605M	None	0	0	0	0	0	0	2.0	2.5	2.7	2.2	<10	80
	F226100F	None	0	0	0	0	0	1.0	2.5	2.3	0	0	<10	2,560
	F21563F	None	0	0	0	0	1.7	2.4	2.0	2.3	3.0	1.7	<10	1,280
	F21550M	None	0	0	0	0	0	1.3	1.3	1.0	1.8	1.6	<10	320

[a] All monkeys were challenged with 5 log units of DEN2 virus (S16803) PDK10, with the exception of monkeys F22605M and F226100F, which received 4 log units of challenge virus (pilot study). The day of challenge was considered day 1 for comparative purposes.
[b–d] See Table 7, footnotes *b* to *d*.

TABLE 9. Protection of monkeys immunized with different ChimeriVax-DEN1–4 formulations and challenged with heterologous WT DEN3 virus[a]

Group	Monkey	Vaccine formulation	Viremia by day postchallenge:										$PRNT_{50}$	
			2	3	4	5	6	7	8	9	10	11	Prechallenge[c]	Postchallenge[d]
9	F21343M	5,5,5,5	0[b]	0	0	0	0	0	0	0	0	0	320	20,480
	F212105F	5,5,5,5	0	0	0	0	0	0	0	0	0	0	640	5,120
	F21339M	5,5,5,3	0	0	0	0	0	0	0	0	0	0	1,280	10,240
	F20788F	3,3,3,3	0	0	0	0	0	0	0	0	0	0	640	40,960
	F21522M	3,3,3,3	0	0	0	0	0	0	0	0	0	0	640	2,560
	F21565F	3,5,5,3	0	0	0	0	0	0	0	0	0	0	1,280	10,240
10	F22127M	None	0	1.6	1.0	1.5	1.6	1.7	0	0	0	0	<10	>20,480
	F20992F	None	0	1.7	1.3	0	1.3	2.5	0	0	0	0	<10	2,560
	F22607M	None	0	0	0	1.7	0	1.0	2.0	1.7	0	0	<10	10,240
	F22606M	None	0	1.7	0	0	0	0	0	2.5	2.0	2.0	<10	10,240

[a] All monkeys were challenged with 5 log units of DEN3 virus (D3, CH53489, PS), with the exception of monkeys F22127M and F20992F, which received 4 log units of challenge virus (pilot study).
[b–d] See Table 7, footnotes *b* to *d*.

neutralizing-antibody titers (at 1 month) were measured postchallenge.

All control animals developed viremia following challenge. All prevaccinated animals were protected (no detectable viremia) against challenge with DEN2 or DEN3 virus, whereas 83% (five of six) were protected against DEN1 or DEN4 virus challenge (Table 11). Animal F21149M (unprotected against DEN1 virus challenge) developed viremia for 4 days, with a peak titer of 3.3 $\log_{10}$ PFU/ml, whereas animal F20977F (partially protected against DEN4 virus challenge) developed a brief viremia with a peak titer of 1.7 $\log_{10}$ PFU/ml (Tables 7 and 10). Both unprotected monkeys had low levels of neutralizing antibody against the challenge virus. The $PRNT_{50}$ of unprotected (F21149M) and partially protected (F20977F) monkeys prior to challenge were 20 and 40 against DEN1 and DEN4 viruses, respectively (Tables 7 and 10). Neither monkey had become viremic to ChimeriVax-DEN1 or ChimeriVax-DEN4 virus postimmunization (Table 5). All monkeys developed high levels of neutralizing antibodies post-challenge, which were significantly higher (three- to five-fold) than those of control groups with the exception of DEN4 virus (Table 11).

DISCUSSION

Genomic stability and mouse neurovirulence. ChimeriVax-DEN1–4 viruses were constructed by inserting prME genes of WT DEN viruses into core and nonstructural genes of YF 17D virus. On transfection of Vero cells with chimeric RNA transcripts, progeny viruses were harvested from supernatants (P1), amplified once to produce P2, and subjected to three rounds of direct plaque-to-plaque purification to produce the final cloned PMS (P7) viruses. Three further passages under cGMP were required to produce VL (P10) viruses. Due to the presence of quasi-species viruses generated by error-prone RNA-dependent RNA polymerase during the replication cycle of RNA viruses, it was necessary to sequence chimeric viruses at various passage levels to monitor their genetic stability. Before final amplification of P6 to produce PMS at P7, vaccine viruses were sequenced and compared to the original consensus sequences. Only clones with authentic sequences were further processed to vaccine stocks. P7 viruses were also passaged up to 13 times in Vero cells (i.e., 10 passages beyond the vaccine level) to determine their genetic stability. The degree of genetic stability differed between chimeras; ChimeriVax-

TABLE 10. Protection of monkeys immunized with different ChimeriVax-DEN1–4 tetravalent formulations and challenged with heterologous WT DEN4 virus[a]

Group	Monkey	Vaccine formulation	Viremia by day postchallenge:										$PRNT_{50}$	
			2	3	4	5	6	7	8	9	10	11	Prechallenge[c]	Postchallenge[d]
11	F21311M	3,3,3,3	0[b]	0	0	0	0	0	0	0	0	0	640	1,280
	F209108F	3,3,3,3	0	0	0	0	0	0	0	0	0	0	80	1,280
	F21355M	3,5,5,3	0	0	0	0	0	0	0	0	0	0	320	640
	F213114F	5,5,5,5	0	0	0	0	0	0	0	0	0	0	320	2,560
	F21534M	3,5,5,3	0	0	0	0	0	0	0	0	0	0	20	5,120
	F20977F	3,5,5,3	0	0	0	0	0	1.7	0	1.7	0	0	40	5,120
12	F22612M	None	0	0	0	0	1.0	0	1.5	2.3	2.5	3.1	<10	2,560
	F207107F	None	0	0	0	1.0	2.0	2.9	2.7	2.0	0	0	<10	5,120
	F21752M	None	0	0	0	2.2	3.2	3.6	2.9	0	0	0	<10	2,560
	F21102F	None	0	0	0	1.7	2.4	2.6	3.3	2.9	2.4	1.8	<10	5,120

[a] All monkeys were challenged with 5 log units of DEN4 virus (Carib, 341750, nonattenuated), with the exception of monkeys F22612M and F207107F, which received 4 log units of challenge virus (pilot study).
[b–d] See Table 7, footnotes *b* to *d*.

TABLE 11. Protection of monkeys against challenge with WT DEN1 to DEN4 viruses in cynomolgus monkeys immunized s.c. (~ 6 months previously) with one dose of ChimeriVax-DEN1–4 GMP vaccine formulation

Group	Previous CV DEN1–4	Challenge virus[a]	Strain of challenge DEN virus	No. viremic/ total no.	Mean viremia		% protected[b]	GMT $PRNT_{50}$	
					Peak (log_{10} PFU)	Duration (days)		Prechallenge	Postchallenge
5	Yes	DEN1	West Pacific 74	1/6	1.7 (3.3)[c]	0.7 (4.0)[c]	83	63	14,481
6	No	DEN1		4/4	3.2	6.0	0	<10	3,044
7	Yes	DEN2	S16803	0/6			100	30	1,437
8	No	DEN2	PDK-10	4/4	2.5	4.5	0	<10	538
9	Yes	DEN3	CH53489	0/6			100	718	10,240
10	No	DEN3		4/4	2.2	4.2	0	<10	2,229
11	Yes	DEN4	341750 Caribbean	1/6	0.6 (1.7)[c]	0.3 (2)[c]	83	127	2,032
12	No	DEN4		4/4	3.2	5.2	0	<10	3,620

[a] Two animals in each of groups 6, 8, 10, and 12 received 4 log units of challenge virus (in the pilot study); all other animals received 5 log units of appropriate challenge virus (main study).
[b] No virus could be found in a plaque assay in Vero cells when using undiluted or 1:2 or 1:10 dilutions of sera obtained from days 2 to 11 postchallenge.
[c] Viremic animals only.

DEN1 and ChimeriVax-DEN2 acquired more mutations than did ChimeriVax-DEN3 and ChimeriVax-DEN4 when passaged in Vero cells. Moreover, the plaque-purified viruses (used for vaccine production) accumulated fewer mutations than did their uncloned counterparts (45). It is possible that a large number of mutations are introduced by errors in Sp6 RNA polymerase during in vitro RNA transcriptions, which are eliminated by plaque purification. Generally, the appearance of mutations coincided with increases in viral titers. Such "adaptive mutations" reoccurred at similar positions (i.e., amino acids 202, 204, and 251, in the E gene and amino acid 177 in the NS4b genes) within genomes of different chimeras (45). A number of mutations were reported for the NS4b gene of a recombinant DEN4 vaccine candidate following Vero cell passage (2), indicating the importance of NS4b protein in replication and/or assembly of infectious DEN viruses.

The appearance of mutations in DEN virus chimeras resulted sometimes in a change in plaque size or neurovirulence of the virus for infant mice. The E204 K-to-R substitution in the DEN1 virus envelope, for example, increased the plaque size and reduced its neurovirulence for infant mice and viscerotropism and viremia for nonhuman primates (Guirakhoo, unpublished), whereas mutations in DEN2 and DEN3 virus genomes did not appear to have a major impact on the neurovirulence of the vaccine viruses. Because the vaccine lot of DEN1 virus chimera had acquired an attenuating mutation, it was necessary to further evaluate the impact of this mutation in additional relevant animal models, such as monkeys and mosquitoes. The viscerotropism and neurovirulence of mutant and WT DEN1 virus chimeras were compared in monkeys inoculated by the s.c. or i.c. routes. These viruses were also evaluated for their replication in mosquito vectors by infection of *Aedes aegypti*, the principal mosquito vector for DEN viruses, by the intrathoracical route (Guirakhoo, unpublished). The results provided reassurance that ChimeriVax-DEN1 mutant vaccine will remain safe in the human host and will not replicate in mosquitoes, even if it reverts to the WT sequence in a vaccinated individual.

The suckling-mouse neurovirulence test was adopted as a quality control release test for all vaccine lots because it had been shown to detect minor changes in the genome of ChimeriVax-JE (37) or ChimeriVax-DEN chimeras that are associated with neurovirulence. The neurovirulence of the DEN1 virus chimera was significantly reduced from P7 to P10 (vaccine level), and the virus remained attenuated up to P20 in Vero cells. No change in neurovirulence was observed for the other chimeras (DEN2 to DEN4 viruses) between PMS (P7) and bulk vaccines (P10). Extensive passaging to P20, however, produced viruses which were generally less neurovirulent than their P7 parents. In the case of the DEN1 virus chimera, this was obviously due to the E204 mutation. For other chimeras, however, slight reduction in neurovirulence for infant mice could not be supported by a change in the virus genome (e.g., DEN4 virus did not accumulate any mutations based on consensus sequencing). It is possible that subpopulations of mutants (estimated to be $\leq$10% of the total virus population) with reduced neurovirulence for infant mice are present, which cannot be detected by consensus sequencing. Alternatively, viruses could accumulate cellular proteins by in vitro passages, which may reduce their replication in suckling-mouse brains.

Our data are in agreement with other studies which showed that chimerization using attenuated backbones such as YF 17D (5, 8, 14–16, 48), DEN2 (4) or DEN4 (49) virus is generally sufficient to produce vaccine candidates for dengue (nonencephalitic). To produce chimeric vaccine viruses for encephalitic flaviviruses, it seems that further attenuations, usually within but not limited to the E genes, may be necessary. Successful vaccine candidates were produced using attenuated E genes of Japanese encephalitis (JE) (SA14-14-2-strain) (7), West Nile (1) and St. Louis encephalitis (K. Pugachev, submitted for publication) viruses within the YF 17D backbone or West Nile and Langat viruses within the DEN4 virus backbone (43, 44).

Monkey neurovirulence studies. The YF 17D vaccine was first used for human immunization in 1936. Over the past 60 years, 400 million people have received the vaccine, with a remarkable record of safety and efficacy. However, recent reports estimate a very low incidence (~1:250,000) of YF 17D vaccine-associated viscerotropic disease and encephalitis (6, 31). In dengue, involvement of the central nervous system is an equally rare event. Reports of patients with a diagnosis of DEN virus encephalitis indicate that the virus very rarely crosses the blood-brain barrier and targets neuronal cells (21, 29). Most neurological adverse events appear to be due to

encephalopathy in DHF/DSS caused by edema or electrolyte imbalance. Because ChimeriVax-DEN viruses contain the replicative machinery of YF 17D together with E genes of DEN viruses, it is possible that similar viscerotropic and encephalitis events could occur with this vaccine. Since these events are rare, they cannot be studied in early clinical trials. However, animal models can be used in preclinical studies to predict safety of the vaccine candidates for humans. The neurovirulence and viscerotropism of ChimeriVax-DEN1–4 tetravalent vaccine were determined in a formal neurovirulence test performed as specified by the WHO protocol (50). Prior to this test, it was necessary to demonstrate that these viruses can independently replicate in vivo and that neurovirulence of the "hotter" viruses (DEN1, DEN2, and DEN4 viruses) is not masked by that of the "colder" (DEN3 virus) one. Inoculation of a tetravalent mixture of four chimeric viruses into brains of suckling mice demonstrated that neurovirulence and AST of a tetravalent mix is similar to that of the "hotter" monovalent components. The independence of replication of the four chimeras was also demonstrated in mosquito vectors (22).

The potential neurovirulence and acute toxicity of the ChimeriVax-DEN1–4 tetravalent vaccine was compared with those of YF-VAX in the monkey neurovirulence test. i.c. injection of tetravalent DEN virus vaccine and YF-VAX was well tolerated at doses of ~4.1, ~4.3, ~4.9, and ~5.0 $\log_{10}$ PFU for ChimeriVax-DEN1 to ChimeriVax-DEN4, respectively, within the tetravalent vaccine, and ~4.7 $\log_{10}$ PFU for YF-VAX. Vaccine-related clinical signs of pupil dilation observed between days 16 and 31 were more frequent in the YF-VAX-treated group, especially on days 17 and 18, when clinical scores were significantly different between groups. Vaccine-related gross anatomic and histological findings from nonneural tissues were secondary to vaccine-induced immunostimulation. Peak total viremia titers and number of viremic days were greater in monkeys treated with the tetravalent DEN virus vaccine compared to those in YF-VAX-treated monkeys. Nevertheless, viremia levels for both groups remained within acceptable limits for individual monkey and group titers established under the WHO requirements for the YF 17D vaccine (50). All monkeys seroconverted following i.c. inoculation of tetravalent vaccine, and none had a detectable cross-reactivity with YF 17D virus in the LNI assay. Antibody levels against the DEN1 to DEN4 virus serotypes did not significantly vary within the tetravalent vaccine ($P > 0.15$ by ANOVA), despite higher viremia levels detected for DEN3 and DEN4 virus serotypes. Viremia, which reflects the release of viruses from infected cells into the bloodstream and rapid clearance by the reticuloendothelial system (40), was measured for determination of the viscerotropism of viruses. However, since the tissue tropism of ChimeriVax-DEN viruses is unknown at present, daily measurements of viremia may not precisely reflect the dynamics of virus replication in the host. The neurovirulence of the tetravalent DEN virus vaccine preparation was minimal, and the target region scores were significantly lower than the scores for the group treated with YF-VAX ($P < 0.05$). Clinical and histological lesion scores induced by the YF 17D vaccine were similar to those reported for the YF 17DD substrain (30). These data are also consistent with our previous observations with ChimeriVax-JE (39) or ChimeriVax-DEN2 vaccines (data not shown), in which the mean lesion scores for monkeys treated with chimeras were significantly lower than the scores for the group given the YF 17D vaccine. Thus, the tetravalent formulation met or exceeded the safety test requirements for the YF 17D vaccine. It would be interesting to see if other chimeric flavivirus vaccines (based on the YF 17D, DEN2, or DEN4 virus backbone) have similar or better safety profiles when inoculated into monkeys by the i.c. route.

Immunogenicity and protection. Neutralizing antibodies directed against the E protein are considered the principal mediator of immunity against flavivirus reinfection, and the demonstration of neutralizing antibodies is a surrogate of protective immunity. Studies with animal models, principally mice, have demonstrated that the transfer of immune serum raised against E proteins of DEN2, YF 17D (3), and tick-borne encephalitis (42) viruses and monoclonal antibodies against JE virus (52) conferred protection against challenge with WT viruses. The level of protection in immunized mice was directly proportional to the passive titer of neutralizing antibodies. Adoptive transfer of T cells is also effective in preventing illness in recipient mice, indicating that effector T cells can limit the severity of disease by eliminating infected cells. Thus, an effective vaccine should elicit durable neutralizing-antibody responses, as well as strong memory B- and T-cell responses. A live vaccine against DEN virus would therefore be more efficient than inactivated or subunit vaccines, which require multiple doses, do not provide strong memory or durable immunity, and do not efficiently elicit major histocompatibility complex class I restricted T-cell responses.

All monkeys became viremic on inoculation of a single dose of one of four tetravalent formulations. All four serotypes were detected in all four groups, with the exception of serotype 1, which was not detected in any animals of group 2. The magnitude of viremia in the high-dose group formulation (group 1) was similar to that of the low-dose group (group 4) (2.6 and 2.5 log units, respectively). However, the duration of viremia in the low-dose group was longer than in the high-dose group (5.5 and 4 days, respectively). A similar inverse relationship between dose and duration of viremia has been reported previously in monkeys inoculated with YF 17D (10) or in humans vaccinated with ChimeriVax-JE virus (35, 38). The onset of viremia was, however, dose dependent (i.e., early in the high-dose group [days 2 to 7] and late in the low-dose group [days 3 to 11]). It is possible that higher doses of vaccine induce a stronger innate immune response that limits the replication of the virus. When the concentration of one or two components in a tetravalent formulation was reduced, the corresponding viruses were either undetectable (e.g., DEN1 virus in group 2) or detected very late (e.g., DEN4 virus in groups 2 and 3). As observed in i.c.-inoculated monkeys, the DEN3 and DEN4 virus chimeras induced viremia with a higher magnitude and duration than did the equivalent concentrations of the DEN1 and DEN2 virus chimeras. Interestingly, the DEN3 and DEN4 virus chimeras maintained the WT envelope genes during vaccine manufacture whereas the DEN1 and DEN2 virus chimeras acquired one or two mutations within their envelope genes that may have contributed to their lower viremia in monkeys. There is a theoretical risk that ChimeriVax-DEN1–4 viruses could infect insects (mosquitoes, biting flies) or ticks feeding on the blood of vaccinated subjects and be transmitted to animals or other humans on refeeding. If secondary transmis-

sion were to occur, there is an associated risk of virus mutation and reversion to virulence. This theoretical risk is considered minimal according to the results of vector studies with mosquitoes (22) and the findings in both monkeys and humans of low viremia titers which were invariably below the threshold for oral infection of mosquito vectors, which is generally in the range of 3.5 to 5.0 $\log_{10}$ PFU/ml (19). It is thus highly unlikely that viremia caused by the tetravalent vaccine in humans would result in mosquito infection.

All monkeys developed neutralizing-antibody responses to all four DEN WT virus serotypes in the high- and low-dose groups (groups 1 and 4, respectively). The GMTs in the high-dose group animals, measured on days 31 and 121 (data not shown) postimmunization, were higher than those in the low-dose group. When the concentration of the DEN4 (but not DEN3) virus component was reduced, three monkeys in group 2 and two monkeys in group 3 did not seroconvert to the DEN4 virus. Since both DEN3 and DEN4 virus chimeras contain authentic WT envelopes, it is possible that in a tetravalent formulation, similar doses of both viruses need to be present to avoid any dominance or interference in terms of immunogenicity. Interestingly, when empirically derived live attenuated DEN virus vaccines (LAV) were inoculated into humans, the dominant virus in terms of viremia and neutralizing-antibody response was the DEN3 virus component (23, 47). It is likely that the DEN3 LAV was either underattenuated or accumulated quasispecies of viruses with a WT envelope.

To assess the protective efficacy of tetravalent vaccine formulations, monkeys were randomly reassigned to four new groups at 6 months postimmunization, and each group was challenged with one WT heterologous serotype. All vaccinated monkeys were protected (as shown by the lack of viremia postchallenge), with the exception of two animals (one in the DEN1 and one in the DEN4 virus challenge group), whereas all control group animals (naive animals) became viremic. At the time of challenge, unprotected animals had $PRNT_{50}$ of 20 and <10 against the DEN1 and DEN4 viruses, respectively. The unprotected monkey (F21149M) in the DEN1 virus group had not become viremic to DEN1, DEN3, or DEN4 virus chimeras and showed only a single viremia day (day 10) for DEN2 virus after vaccination. Consequently, this monkey was seronegative ($PRNT_{50}$, <10) for the DEN3 and DEN4 virus chimeras and had developed a very low titer ($PRNT_{50}$, 1:10) for the DEN1 virus by day 31 postimmunization. Similarly, monkey F20977, which was only partially protected against DEN4 challenge, had not become viremic for the DEN1 or DEN4 virus chimeras postvaccination. It was seronegative for DEN4 and borderline positive ($PRNT_{50}$, 10) for DEN1 virus 1 month postimmunization. However, both DEN2 and DEN3 virus serotypes had been detected for several days in this monkey, which resulted in the development of high levels of neutralizing antibodies ($PRNT_{50}$, of 2,560 and 320 against DEN2 and DEN3 viruses, respectively). These data suggest that in general, a serum $PRNT_{50}$ of >20 can protect the host against a high dose (5 $\log_{10}$ PFU) of a challenge virus. The actual protective titer may even be lower than 1:20, considering that in natural infections only ~3 log units of virus may be inoculated into the host by a mosquito vector. Moreover, there might be cases in which an individual host can be protected with a serotype specific $PRNT_{50}$ of ≤20 (i.e., monkey F212117F, F21544M, and F21534M). This may indicate that other factors, such as the presence of DEN virus cross-neutralizing antibodies (25) or T-cell memory, may contribute to the protection of tetravalent vaccine-inoculated humans against dengue disease. It is possible that the challenge virus that enters the cells but escapes neutralization by antibodies is eliminated by a second line of defense provided by cytotoxic T lymphocytes. The E proteins of DEN virus (27), as well as capsid (11) and nonstructural (NS) proteins (28, 32) expressed by infected cells, serve as targets for cell killing by cytotoxic T lymphocytes. It is also important to mention that there was no enhancement of infection in the two unprotected animals. This could be an important safeguard in the application of a tetravalent vaccine, where a low level of antibody to one serotype is thought to be implicated in developing DHF/DSS by antibody-dependent infection enhancement phenomena. The levels of neutralizing antibodies postchallenge were significantly higher than those prechallenge or in control groups, indicating an immunological memory provided by B and T cells having high-affinity antigen receptors in immunized monkeys.

The ChimeriVax technology offers a good probability of successful DEN virus vaccine development. The vaccine attributes include the potential for single-dose application, absent or minimal reactogenicity, durable immunity, reduced potential for interference between the individual components in a tetravalent formulation, and low cost of manufacture. Based on our data, the ideal tetravalent formulation(s) for humans may contain equal amounts of each of four ChimeriVax-DEN virus serotypes (e.g. 5,5,5,5 or 4,4,4,4 $\log_{10}$ PFU) and could be administered twice, e.g., at a 6-month interval, similar to LAV vaccine (47), to ensure 100% seroconversion.

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